THIMET OLIGOPEPTIDASE 1 AND THIMET OLIGOPEPTIDASE 2 ARE REDOX-SENSITIVE PEPTIDASES INVOLVED IN PLANT IMMUNITY AND OXIDATIVE STRESS RESPONSES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2017

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Cornell University 2017

Plant hormones are molecules with vital regulatory functions in growth, development, and defense signaling. Major components of biotic and abiotic stress responses are reliant on plant hormone signaling. The plant hormone salicylic acid (SA) has a crucial role in mounting and orchestrating plant defense responses. Thimet oligopeptidase 1 (TOP1) and thimet oligopeptidase 2 (TOP2), are two metallopeptidases capable of interacting with SA. The characterization of TOP function has elucidated roles in plant immunity and oxidative stress responses. We investigated multiple aspects of TOP regulation. We have determined that TOPs form redox-sensitive oligomers. Additionally, our findings suggest that the cellular redox-regulator, glutathione, regulates TOP oligomerization. Furthermore, the activities of TOPs are modulated by thiols. In addition, we examined the expression profile of SA-binding proteins in plant immune-related conditions. We identified five genes encoding putative SABPs that are co-expressed and upregulated during biotic stress responses and SA-regulated processes.

BIOGRAPHICAL SKETCH

Timothy Westlake was born and raised in central Massachusetts. Timothy is an active volunteer in his community; he is a continual volunteer at the HealthAlliance Hospital that he was born in. Timothy attended University of Massachusetts (UMASS) Lowell to pursue a degree in Biology. At UMASS, Timothy was elected vice president of biology club for his final two years of tenure. Timothy became a student assistant in the university's microbiology laboratory where he instructed students in techniques related to microbiology. As an undergraduate, Timothy directed a National Science Foundation (NSF) funded video about climate change. He was a volunteer for the Friends of Wachusett research program at the Wachusett Mountain State Reservation.

Timothy came to Cornell University with support of the SUNY Diversity Fellowship. During the majority of his time at Cornell, he was a student of Professor Sorina Popescu and Professor Jian Hua. Teaching has always been a passion of Timothy. During his time at Cornell, Timothy mentored three students in the NSF-funded Plant Genome Research Program (PGRP) summer internship program. He was a teaching assistant for two semesters for the fantastic course Magical Mushrooms, Mischievous Molds. Recently, Timothy was awarded the NSF East Asia and Pacific Summer Research Institute (EAPSI) fellowship and spent 3 months researching in Japan. During this time, Timothy joined the RIKEN Plant Immunity Research Unit.

Timothy has a wide range of hobbies related to traveling and sightseeing. During his time at Cornell, he has traversed the gorges and waterfalls of Upstate New York, and explored the parks and arboretums. Timothy is an avid fan of nature, wild-life, and animals. He is a committed supporter of animal rights.

Dedicated to my grandmother Ruth Westlake (March 24, 1929-December 2, 2016)

ACKNOWLEDGMENTS

I would like to extend gratitude to my advisors, Professor Sorina Popescu and Professor Jian Hua for their support during my graduate studies. Professor Popescu provided me with unwavering support during my experiments and creating a lab that felt like home. I thank her for allowing me to join her laboratory. It has been a fantastic opportunity to work alongside Professor Popescu and her laboratory members. I am also very grateful to Professor Hua for advising me on thesis matters as well as my research.

I thank my committee member Professor Stephen Winans for his helpful feedback and advice during the course of the project. He is a great professor and instructor.

I would like to acknowledge all the members of the Popescu laboratory for their contribution to the project. I thank Elizabeth Brauer for her friendship, mentorship, protocols and help with experiments. She was a fantastic laboratory mate and collaborator. Her vast knowledge in the field of plant pathology was extremely helpful and inspiring. There was nothing more I could have asked for in a laboratory mate.

I wish to acknowledge the contributions of Giulio Zampogna. He was a fantastic laboratory partner and a major contributor to our knowledge of plant TOPs. I greatly enjoyed our work together. I acknowledge our combined efforts in exploring the functions of TOPs in plants. He is an extremely hard worker. We endured extremely long time point experiments and spent well over 24 hours at a time working diligently in pathogen experiments. Through our combined work, we characterized the involvement of TOP's in plant immunity. He is a great researcher.

I wish to thank Austin Lee for his contributions to this project. He was a dedicated student research assistant. He continually showed strong commitment to our work. His willingness to work strenuous hours has always been appreciated. We are grateful for his help with germination assays, cell death assays, infections, QRTPCR, genotyping, sampling, and many other experiments. His independent assay setup and data collection was important to our work and results. I thank Nabil Elrouby for his insight, help with the project, and his friendship. It was a fantastic opportunity to work alongside him. His vector library was a valuable asset to the experiments. I would also like to extend gratitude to Dharmandra Singh for his protocol and advice on the protoplast experiments. His friendship was invaluable to me over the years. I would also like to acknowledge our former laboratory manager Mauricio Calvino. He is an extremely dedicated researcher and a good friend.

I would also like to express my gratitude to the summer interns Anna Knapp, Julia Wang, and William Ricci. It was a fantastic opportunity to mentor these amazing students. I am very proud of all my interns and their accomplishments. It is fantastic to hear about their current academic pursuits. Anna Knapp was the first high school intern I mentored. She is a fantastic student and showed strong commitment to the project. I was thrilled to hear about her undergraduate pursuits in science and

engineering. I thank Julia Wang for her dedication to her summer project. Her time and efforts put into the project was appreciated. My undergraduate intern, William Ricci, is among the most talented workers I have had the chance to work with. His untiring dedication to this project was immensely helpful. The work he put into the project was published. His commitment to strenuous hours of work was much appreciated. I am thankful for his contributions during and outside the program. His results pertaining to the enzyme assays and protoplast experiments greatly contributed to the characterization of TOPs. I am grateful for the experiments and data collection performed by the interns. Their work with the germination assays, oxidative stress assays, pathogen assays, and other experiments were greatly appreciated.

I acknowledge the contributions of Amanda Gray to the research project and editing of the thesis. Her tireless dedication has made her an extremely valuable collaborator throughout this project. It is difficult to finds words to express my gratitude for her help throughout the years. I appreciate her immense support during the duration of the project. She has been a valuable asset to this project and even greater friend.

I thank Ken Shirasu's Plant Immunity Research Group. I greatly appreciate the feedback and help with my project. The laboratory group is among the hardest and most dedicated researcher group I have ever worked with. I thank Nobuaki Ishihama for his mentorship and help with protocols and instrumentation. He was immensely helpful to this project. I am honored to have had the opportunity to work with members of this laboratory.

I thank Joi-Anne Strauss, Helene Dillard, and Mary McKellar for their help in obtaining necrotrophic pathogens.

I thank Professor George Hudler. It was a fantastic opportunity to be a teaching assistant for his course. He is beloved by the students of Cornell University for a reason.

I would like to thank Murli Manohar and Hyong-Woo Choi for their immense help with size-exclusion chromatography, gel filtration, protein-purification, and protein-production. Their instruction and mentorship was greatly appreciated. I am very grateful for their help and friendship.

I would like thank Gary Horvath for his contributions in preparing the media/buffers/agars/solutions. He was an extremely reliable worker and extremely helpful to this project.

I would also like to thank the Boyce Thompson Institute's greenhouse staff for their assistance throughout the project.

Lastly, I thank Boyce Thompson Institute for Plant Research, Cornell University, and RIKEN for hosting my research.

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LIST OF ABBREVIATIONS

A. thaliana Arabidopsis thaliana

A.U. arbitrary units

AA Amino Acid

AtMIF1 *Arabidopsis thaliana* Macrophage migration inhibitory factor 1

AtTN3 *Arabidopsis thaliana* TIR-nucleotide binding site 3

Avr Avirulent

Azidosalicylic acid AzSA

C Cysteine

Ca Calcium

CML42 Calmodulin-like 42

CFU colony forming unit CFU

DPI days post-inoculation DPI

DNA Deoxyribonucleic acid

EDS Enhanced Disease Susceptibility

ETI Effector Triggered Immunity

Flagellin-sensitive 2 (FLS2)

Fv/Fm MAXIMUM QUANTUM YIELD

GFP Green fluorescent protein

GSH Glutathione (reduced)

GSSG Glutathione (oxidized)

HEXXH His-Glu-Xaa-Xaa-His

His Histine Tag

HR Hypersensitive Response

IPL Isochorismate pyruvate lyases

IS Isochorismate

ISC Isochorismate synthase

Kings's B (KB)

LECRK Lectin Receptor-Like Kinase

LRR Leucine Rich Repeat

Luc Luciferase

LB Lysogeny broth LB

MHC Major histocompatibility complex

MAMP Microbe Associated Molecular Pattern

MS Mass Spectrometry

MBL Mannose Binding Lectin

MBL-SA Mannose Binding Lectin-SA

MIF Macrophage Inhibitory Factor

MKK MAP Kinase Kinase

MPK MAP Kinase

MTI Microbe associated molecular pattern triggered immunity

MV Methyl Viologen

National Institute of Health (NIH)

NBS Nucleotide Binding Site

NLR Nucleotide-binding/leucine-rich

NPR Non-expressor of pathogenesis related protein

P. carotovurm Pectobacterium carotovorum

PAL phenylalanine ammonia-lyase

PCD Programmed Cell Death

phytoalexin deficient 4 (PAD4)

PR1 pathogenesis related protein 1

PRR Pattern Recognition Receptor

Pst Pseudomonas syringae pv. *Tomato*

Quantitative Real Time PCR (QRTPCR)

R resistance

RIN4 (RPM1-interacting protein 4)

RLK Receptor-Like Kinase

RLU Relative Luminesce Units

RNA Ribonucleic acid

RNA-Seq RNA Sequencing

RNS Reactive Nitrogen Species

ROS Reactive Oxygen Species

RPS Resistance to *Pseudomonas syringae*

RRS Resistance to *Ralstonia solanacearum*

S. sclerotiorum Sclerotinia sclerotiorum

SA Salicylic Acid

SABP Salicylic Acid Binding Protein

SAR Systemic Acquired Resistance

SLCA split luciferase complementation assay

TIR Toll/interleukin receptor

TIR-NBS-LRR toll-interleukin receptor-nucleotide binding-leucine-rich repeat

TOP Thimet Oligopeptidase

TOPL TOP-Like Protein

UV Ultra Violet

Y2H Yeast Two-Hybrid

LIST OF SYMBOLS

° degree

CHAPTER 1

INTRODUCTION

1.1 Salicylic Acid

Plants are sessile organisms; perception and responses to environmental stimuli is necessary for successful growth and survival. Plant hormones are signaling molecules with vital regulatory functions in plant growth and development, as well as in biotic and abiotic stress responses (Santner et al., 2009; Vlot et al., 2009). These signaling molecules are synthesized through various metabolic pathways and are perceived by plant cells to induce physiological changes (Santner et al., 2009). Hormones elicit a cascade of signaling events, transcription reprogramming, and protein modifications, which facilitate responses to environmental stressors and regulate aspects of plant development (Pieterse et al., 2012; Santner et al., 2009; Vlot et al., 2009).

Salicylic acid (SA) is a phenolic molecule with critical importance in plant physiology, plant development, and plant disease resistance (Vicente and Plasencia, 2011; Vlot et al., 2009). While plant phenolic compounds were traditionally deemed as only secondary metabolites, the role of SA as a plant hormone has been established in the past quarter century. Previously, phenolic compounds were not seen as essential mediators of plant development and physiology (Dempsey et al., 2011; Vlot et al., 2009). Instead, phenolic compounds were thought as non-essential compounds or mere waste products from metabolic pathways (Dempsey et al., 2011; Vlot et al., 2009). Prior to its classification as an endogenous plant signal, the categorization of a phenolic compound as a plant hormone was a subject of controversy. While SA is typically seen today as an immune signal, it was not the hormone's role in biotic defenses responses that cemented the phenolic molecule's classification as a hormone. Interestingly, the discovery of SA's role in flowering and thermogenesis pioneered the importance of SA signaling and provided the first decisive evidence that SA functions as a plant hormone (Dempsey et al., 2011; Khurana and Cleland, 1992; Raskin et al., 1990; Vicente and Plasencia, 2011; Vlot et al., 2009).

SA is a regulator of biochemical processes in plants and is intricately involved in plant defense responses. The hormone mediates biotic defense responses towards biotrophic pathogens and hemibiotrophic pathogens. While traditionally seen as an immune signal for biotrophic pathogens (McDowell and Dangl, 2000), increasing evidence suggest that SA also mediates defense responses to certain necrotrophic pathogens. SA and SA-analogs increase resistance to necrotrophic pathogens like *Botrytis cinerea*. (Achuo et al., 2004; Ferrari et al., 2003). Furthermore, certain necrotrophic pathogens can target and degrade SA-derivatives (Penn and Daniel, 2013).

SA has a central role in multiple layers of plant immunity, as the hormone regulates both local and systemic immune responses (Lawton et al., 1995; Vlot et al., 2009). In response to pathogen infection, salicylic acid levels drastically increase, resulting in the induction of plant biotic defense responses. SA also plays a major role in abiotic defense responses (Vicente and Plasencia, 2011). The hormone function has been implicated in oxidative stress responses, resistance to heavy metal toxicity, cold stress responses, heat stress responses, and osmotic stress responses (Jayakannan et al.. 2015; Khan et al., 2013; Metwally et al., 2003; Vicente and Plasencia, 2011; Vlot et al., 2009). Also, SA has important regulatory functions in an array of plant developmental stages such as germination, flowering, and senescence (Morris et al., 2000; Vicente and Plasencia, 2011; Vlot et al., 2009).

Researching SA is of the utmost importance. There is a rising need to bolster plant defenses to feed the growing population. Understanding SA signaling is instrumental in mounting a multi-faceted approach to enhancing resistance to environmental stressors. Furthering the knowledge of plant hormone signaling is a necessity for the future development of stress-resistant crop lines.

1.12 Salicylic Acid Mediated Immune Responses

SA signaling is intimately involved in microbe-associated molecular patterns (MAMP)-triggered immunity (MTI), effector-triggered immunity (ETI), and in systemic acquired resistance (SAR). Activation of MTI occurs in response to the perception of conserved MAMP ligands by plant pattern recognition receptors (PRR).

MAMPs are conserved structures that have essential functions in pathogens' biology. MAMPs are typically integral structural components of the microbes, such as chitin, lipopolysaccharides, sterols, and glycans (Kaku et al., 2006; Newman et al., 1995; Newman et al., 2013). In addition, important macromolecular structures such as flagellin and have been identified as MAMPs (Chinchilla et al., 2006; Felix et al., 1999). The activation of MTI typically starts on the plasma membrane through the action of receptor-like kinases (RLK) (Newman et al., 2013). RLKs are plasma membrane PRR proteins (Greeff et al., 2012; Newman et al., 2013). The extracellular domain of an RLK functions in ligand binding (Greeff et al., 2012; Newman et al., 2013). Ligand binding constitutes the first steps in MAMP recognition (Greeff et al., 2012; Newman et al., 2013). In the case of the RLK Flagellin-sensitive 2 (FLS2), the extracellular domain directly interacts with flg22, a component of bacterial flagellin (Chinchilla et al., 2006). After the direct interaction, FLS2 is rapidly phosphorylated, driving MTI signal transduction (Schulze et al., 2010). Phosphorylated FLS2 interacts with brassinosteroid insensitive 1-associated kinase (BAK1) and *Botrytis*-induced kinase (BIK1) which induces a cascade of phosphorylation events (Chinchilla et al., 2007; Lu et al., 2010; Newman et al., 2013; Schulze et al., 2010). The cascade of events results in the activation of MAP Kinase (MPK) and MAP Kinase Kinase (MKK) pathways which in turn activate transcriptional regulators of MAMP-triggered genes (Asai et al., 2002; Bethke et al., 2009; Nitta et al., 2014; Suarez-Rodriguez et al., 2007). Defense activation includes the induction of regulators of plant immunity such as *WRK*Y transcription factors (Asai et al., 2002). MTI signaling triggers the induction of plant defense genes, reactive oxygen species production, increased callose deposition, stomatal closure, and increased SA production (Felix et al., 1999; Gomez et al., 2002; Gohre et al., 2012; Melotto et al., 2006; Newman et al., 2013, Yi et al., 2014).

MTI-induced defense responses are tightly associated with SA signaling. SA production induced by MTI is instrumental to local immunity as SA upregulates defense gene expression (Tateda et al., 2014; Yi and Kwon, 2014). SA signaling upregulates PRR expression after MAMP detection and results in increased production

of FLS2 and BAK1 (Tateda et al., 2014). The induction of *FLS2* and *BAK1* expression results in increased sensitivity towards the MAMP, which in turns potentiates and strengthens immune signaling (Tateda et al., 2014). The potentiated signal enhances MTI-triggered immune responses, such as increased deposition of callose (Tateda et al., 2014). In addition, the detection of the bacterial MAMP, flagellin, results in an oxidative burst that is, in part, regulated by SA (Yi and Kwon, 2014). Impaired SA production dampened MTI response as the expression of MAMP-triggered genes are reduced but not completely diminished (Tsuda et al., 2008). For instance, the induction of *WRKY29*, a MAMP-triggered immune regulator, is drastically reduced in plants with mutations in the SA biosynthetic pathway (Yi and Kwon, 2014). However, *WRKY29*, is still induced in the absence of SA (Yi and Kwon, 2014).

To subvert MTI, pathogens secrete effectors that suppress immune responses. The functions of effectors are highly diverse and employ various strategies to promote infection (Toruno et al.,2016). Effector targets include major mediators of MTI such as RLK complexes and MAP kinases (Shan et al., 2008; Zhang et al., 2007; Zhang et al., 2012). *Pseudomonas syringae*, a hemibiotrophic pathogen, is a model used to study plant immunity. *P. syringae* effector AvrPtoB interacts with BAK1 to impede FLS2 interactions and impair MTI signaling (Shan et al., 2008). *P. syringae* effector HopAI1 interferes with MAP Kinase signaling by dephosphorylating members of the MAP Kinase signaling cascade (Zhang et al., 2007; Zhang et al., 2012). The repression of MTI signaling results in impaired plant immunity and SA signaling (Tanaka et al., 2015). To counter pathogen effectors, plants evolved ETI. In contrast with MTI, ETI is a rapid and vigorous defense response (Cui et al., 2015). Hallmarks of ETI include a strong ROS burst and a form of programmed cell death known as the hypersensitive response (HR) (Cui et al., 2015; Pontier et al., 1998). HR activates in response to secreted effectors to limit the spread of the pathogen (Cui et al., 2015).

Detection of effectors occurs during specific recognition events mediated by R-proteins (Glowacki et al., 2011). In general, ETI activation is facilitated by nucleotide-binding/leucine-rich-repeat (NLR) proteins (Cui et al., 2015; DeYoung and Innes, 2006). NLR-related immune activation can occur through direct interactions

with the effector or indirectly by detecting changes of host proteins (Cui et al., 2015; van der Hoorn and Kamoun, 2008; Williams et al., 2014). In *Arabidopsis thaliana,* RPS4 (Resistance to *Pseudomonas syringae* 4) and RRS1 (Resistance to *Ralstonia solanacearum* 1) are an NLR R-protein pair that facilitates the detection of the *P. syringae* effector AvrRps4 (Narusaka et al., 2009; Williams et al., 2014). While the exact mode of action of the RRS1/RPS4 is unclear, AvrRps4's direct binding to RRS1 is thought to constitute the first steps of ETI activation. ETI activation in response to *P. syringae* effector AvrRpt2 occurs indirectly. NLR protein RPS2 (resistapnce to *Pseudomonas syringae* 2) guards host protein RIN4 (RPM1-interacting protein 4) (Mackey et al., 2003). In the absence of RIN4, the RPS2-mediated immune response is activated. AvrRpt2, a secreted protease, targets and cleaves RIN4 (Chisholm et al., 2005; Takemoto and Jones, 2005). The cleavage of RIN4 triggers RPS2-dependent immune responses (Mackey et al., 2003; Takemoto and Jones, 2005).

ETI is highly intertwined with SA-regulated processes and SA signaling. For instance, enhanced disease susceptibility 1 (EDS1) and phytoalexin deficient 4 (PAD4), two key components in SA signaling, represent a node for an array of NLR R-proteins (Falk et al., 1999; Feys et al., 2001; Zhou et al., 1998). SA accumulation during pathogen infection is largely dependent on PAD4/EDS1 (Feys et al., 2001, Zhou et al., 1998). In a positive feedback loop to amplify defense signaling, SA signaling induces the expression of *PAD4/EDS1* (Feys et al., 2001). In the absence of EDS1, toll-interleukin receptor-nucleotide binding-leucine-rich repeat (TIR-NBS-LRR)-mediated ETI signaling is mostly inactive (Aarts et al., 1998; Feys et al., 2001). Unimpeded ETI signaling results in increased SA production and SA-dependent defense gene expression that is necessary to mount a local immune response.

SA is involved in the activation of systemic immunity after the perception of a pathogen (Lawton et al., 1995; Vlot et al., 2009). Systemic acquired resistance (SAR) is an inducible broad spectrum resistance response that occurs systemically in response to infection (Durrant and Dong, 2004). The establishment of SAR is dependent on SA-regulated processes (Lawton et al., 1995; Vlot et al., 2009). Impaired SA accumulation halts SAR and results in increased susceptibility to pathogen

infection (Dempsey et al., 1999; Ryals et al., 1996). However, SA is not the mobile messenger for SAR. Evidence suggest that methyl salicylate, a SA derivative, acts as the mobile signal for SAR (Park et al., 2007). SA is thought to be converted to methyl salicylate which is transported to distal tissue and converted back to SA (Forouhar et al., 2005). The conversion to SA in distal tissue then activates SA-signaling to prime plant immune responses (Forouhar et al., 2005).

1.13 Salicylic Acid Synthesis & Perception

SA is synthesized in plants and bacteria (Garcion and Métraux, 2006). In plants, SA biosynthesis is governed by two major pathways. The majority of immune-related SA synthesis occurs through the isochorismate (IC) biosynthetic pathway (Seyfferth and Tsuda, 2014; Vlot et al., 2009; Wildermuth et al. 2001). The pathway constitutes as much as 90% of SA synthesis during pathogen infection (Vlot et al., 2009; Wildermuth et al., 2001). Utilizing products of the shikimate pathway, chorismate is converted to IC by the isochorismate synthase (ISC) enzyme (Garcion and Métraux, 2006; Seyfferth and Tsuda, 2014; Wildermuth et al., 2001). The products of the reaction are converted to SA by a mechanism that has yet to be elucidated (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). ICS-SA pathway occurs in the chloroplasts (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). Export the plant hormone out of the chloroplasts is regulated by ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) (Serrano et al., 2013). In a similar fashion to plants, SA biosynthesis in bacteria is mediated by ICS. However, SA biosynthesis through the IC pathway is clearer in bacteria. After production of IC in bacteria, the conversion to SA is catalyzed via IC pyruvate lyases (IPL) (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). Interestingly, homologs to bacterial *IPL* are not found in plants, thus, suggesting another unidentified enzyme facilitates IC conversation to SA (Dempsey et al., 2011; Seyfferth and Tsuda, 2014).

SA is also synthesized through the phenylalanine ammonia-lyase (PAL) pathway (Seyfferth and Tsuda, 2014). While the PAL pathway does not contribute as much SA as the IC pathway, the importance of the biosynthetic route cannot be dismissed. In *Arabidopsis*, *PAL* quadra-knockout mutations result in approximately

50% reduced accumulation of SA during certain pathogen infections (Huang et al., 2010). Like the IC pathway, the PAL pathway also uses products derived from the shikimate pathway (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). The starting product of the PAL pathway is phenylalanine (Dempsey et al. 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). Phenylalanine is converted to cinnamic acid by PAL enzymes (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). Cinnamic acid is then processed and eventually converted to coumaric acid or benzoic acid (Dempsey et al., 2011; Vlot et al., 2009). The intermediate products are then converted to SA (Dempsey et al., 2011; Vlot et al., 2009).

NON-EXPRESSOR of PATHOGENESIS-RELATED GENES (NPR) family is a focal point in SA perception and regulation. NPR1, a receptor for SA, is a major mediator of SA signaling (Wu et al., 2012). NPR1 is comprised of a C-terminal transactivation domain that is inactivated by the BTB/POZ domain (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). In the cytosol, NPR1 exists predominately in an oligomeric inactive state. The oligomer is maintained by redox-sensitive disulfide bonds (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). Changes in redox state result in the breaking of the redox-sensitive bonds. Furthermore, SA-induced redox changes activate thioredoxins, which reduce the disulfide bonds of oligomeric NPR1 (Tada et al., 2008). Monomerization of NPR1 facilitates the translocation of the monomeric form to the nucleus (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). Nuclear localized NPR1 can interact with transcription factors which in turn mediates SA transcriptional reprogramming (Dempsey et al., 2011; Seyfferth and Tsuda, 2014; Vlot et al., 2009). Specific TGA leucine zipper transcription factors interact with NPR1 that in turn binds to SA-responsive elements (Johnson et al., 2003; Hermann et al., 2013; Seyfferth and Tsuda, 2014; Weigel et al., 2005). NPR1 is processed and then degraded by the 26S proteasome (Spoel et al., 2009; Seyfferth and Tsuda, 2014). NPR1 degradation and turnover are necessary for SA signaling.

SA binding to NPR1 is thought to promote the deoligomerization of NPR1 (Wu et al., 2012). In addition, SA binding is believed to activate the C-terminal transactivational domain by limiting the repressive effects of the BTB/POZ domain (Seyfferth and Tsuda, 2014; Wu et al., 2012). SA-binding of NPR1 occurs at the transitional metal copper site that is mediated by cysteine residues (*Wu et al.,* 2012). However, NPR1's role as an SA-receptor is a subject of discussion. The cysteine residues thought to be essential for SA binding are not conserved throughout plant species (Seyfferth and Tsuda, 2014).

NPR3 and NPR4 have been found to bind to SA and are postulated to be SA receptors (Fu et al., 2012). In the working model presented by Fu et al., 2012, NPR3 and NPR4 mediate SA-signaling by regulating the degradation of NPR1 (Fu et al., 2012). When SA signaling is not required, NPR4 is thought to facilitate the degradation of NPR1 (Fu et al., 2012). The degradation prevents NPR1-mediated signaling in the absence of SA (Fu et al., 2012). When SA production is induced, SA binds to NPR4 and impedes on NPR1-NPR4 interactions thus allowing NPR1 to accumulate in the nucleus (Fu et al., 2012). NPR1 accumulation then mediates SA transcriptional reprogramming. NPR4 affinity has a high affinity to SA; therefore SA can readily impair NPR4-NPR1 binding (Fu et al., 2012). After local pathogen infection, distal leaves are primed for infection. SA signaling starts to activate in distal tissue as SA prevents the binding of NPR4 to NPR1 (Fu et al., 2012). NPR3's affinity to SA is low. It is postulated that SA only binds to NPR3 at high concentrations (Fu et al., 2012; Seyfferth and Tsuda, 2014). At levels of SA that are conducive to NPR3-SA binding, NPR3 interacts with NPR1 (Fu et al., 2012). The interaction between NPR3 and NPR1 then results in NPR1 turnover and degradation (Fu et al., 2012).

SA was found to interact with enzymes involved in oxidative stress and redox-regulation. However, the functional significance of SA-binding is not well understood. Catalase and ascorbate peroxidase have been shown to be SA-binding proteins (SABPs) (Durner and Klessig, 1995; Sanchez-Casas and Klessig, 1994). SA impedes on the activity of the enzymes. Thus, it is postulated that SA's inhibitory activity on

the enzymes may facilitate ROS accumulation during the oxidative burst (Vlot et al., 2009).

In bacteria, SA derivatives are processed into downstream products. For instance, *Pseudomonas aeruginosa* can utilize SA in siderophore production (Ankenbauer and Cox, 1988). In humans, SA binds to and inhibits cyclooxygenases which are involved in the synthesis of prostaglandins, a group of steroid hormones (Klessig et al., 2016; Vane, 1971; Vane, 2003). In addition, in mammalian systems, SA causes mitochondrial damage by interacting with a mitochondrial ferrochelatase (Gupta et al., 2013). SA binds to the ferrochelatase, which in turns inhibit the enzyme's activity (Gupta et al., 2013). The inhibition of ferrochelatase's activity impedes heme biosynthesis and is thought to contribute to SA's damaging effect on mitochondria (Gupta et al., 2013). SA has also been found to interact with two mammalian kinases. Adenosine monophosphate-activated kinase is an SABP that is activated by SA (Hawley et al., 2012). SA activation contributes to an increase in fat metabolism. SA has also been found to bind to IKK-B kinase which may influence inflammation (Kopp and Ghosh, 1994).

Hormones in plants are thought to have limited targets. Traditional models of hormone signaling assume only one or a small number of receptors for a given hormone. However, large-scale screenings for SABPs have shown a plethora of potential SA-binding proteins (Manohar et al., 2015; Moreau et al., 2013; Klessig et al., 2016). Recently, over 150 proteins with SA-binding capacities have been identified through protein microarrays and mass spectrometry approaches (Manohar; et al., 2015; Moreau et al., 2013). It may be that SA is more promiscuous in cellular targets than other plant hormones. In mammalian systems, SA interacts with an array of enzymes, with evidence suggesting there are even more unknown targets. It is possible that SA exerts its effects on a range of proteins as opposed to just a few receptor proteins. Thus, elucidating the function of SABPs would give light to SA-regulated processes and SA signaling.

1.2 Protein Microarray Identifies Salicylic Acid Binding Proteins in Arabidopsis thaliana

A. thaliana is an excellent model for studying plant biology and plant pathology. The small sequenced genome, mapped chromosomes, natural self-pollination, short-life cycle, diploid, and extensive T-DNA library/insertional mutants, make *A. thaliana* ideal for plant biology and molecular biology. *A. thaliana* is infected by a wide range of crop pathogens and has helped our greatly enhanced our understanding of MTI and ETI (Nishimura and Dangl, 2010).

Protein microarray is a valuable tool for conducting large-scale screens for SABPs (Manohar et al., 2015; Moreau et al., 2013). Thousands of plant proteins can be simultaneously tested for SA binding (Manohar et al., 2015; Moreau et al., 2013). Recently, the technique has been used to identify over a hundred *A. thaliana* proteins with the ability to interact with SA (Manohar et al., 2015; Moreau et al., 2013). Purified plant proteins are immobilized on microarray slides (Manohar et al., 2015; Moreau et al., 2013). Utilizing UV-cross-linkable SA analog, 4-azidosaliclic acid (4-AzSA), proteins printed on microarray slides were probed for SA binding (Manohar et al., 2015; Moreau et al., 2013). Crosslinkable 4-AzSA facilitated the stabilization of SA-binding which allowed the detection of potential weak or transient SA interactions. Thimet oligopeptidase 1 (TOP1) was identified as an SABPs (Moreau et al., 2013). Confirmation of TOP1 binding was performed by traditional SA-exclusion chromatography, SA-competitive binding assays, and surface plasmon resonance (Moreau et al., 2013). In addition, TOP1 homolog, TOP2, has been found to bind to SA albeit at a lower affinity (Moreau et al., 2013).

1.21 Thimet Oligopeptidase

TOPs are zinc-dependent thiol-sensitive metallopeptidases belonging to the M3 family (Pierotti et al., 1990; Orlowski et al., 1983; Orlowski et al., 1989; Tisljar

and Barrett, 1990). M3 peptidases contain a zinc-binding His-Glu-Xaa-Xaa-His (HEXXH) active site motif (Pierotti et al., 1990). The M3 family is ubiquitous, and its constituents are found in prokaryotes and eukaryotes. In general, M3 family metallopeptidases are involved in peptide processing, protein degradation pathways, and clearance of low-weight peptides (Rawling and Barrett, 1995; Tisljar, 1993). In mammalian systems, TOP (EP24.15) is ubiquitously expressed and is present in the most tissues. The subcellular localization of the enzyme is the cytosol, plasma membrane and extracellular space (Crack et al., 1999; Garrido et al. 1999). EP24.15 catalyzes the hydrolysis of small peptides typically shorter than 20 AA and has been found to degrade proteasomal processed products (Tisljar, 1993). In mammalian systems, TOP has a plethora of targets. The substrates of mammalian TOP include bioactive peptides, neuropeptides, and peptide hormones (Dahms and Mentlein, 1992; Orlowski et al., 1989). Gonadotrophin-releasing hormone, bradykinin, angiotensin, and nociception are subject to TOP processing (Dahms and Mentlein, 1992; Orlowski et al., 1989). The peptidase regulates peptide signaling by controlling the accumulation and activity of bioactive peptides (Dahms and Mentlein, 1992; Orlowski et al., 1989). TOP cleavage may inactivate the bioactive peptide and limit the association of the peptide and the respective receptor (Dahms and Mentlein, 1992; Lew et al., 1994; Orlowski et al., 1989). Interestingly, mammalian TOP has been found to interact with hormone receptors (Shivakumar et al., 2005). EP24.15 binds to angiotensin II receptor and bradykinin B2 receptor (Shivakumar et al., 2005). The function of plant TOPs in signaling and the processing of bioactive peptides is unknown.

Significant to disease development, mammalian TOP is implicated in Alzheimer's disease and immunity. The enzyme can cleave full-length amyloid precursor protein and is postulated to have a neuroprotective function (Pollio et al.

2008; Koike et al., 1999; Yamin et al., 1999). Mammalian TOPs function in adaptive immunity (Portaro et al., 1999; Silva et al., 1999). The peptidase has been found to cleave antigenic peptides that interact with the major histocompatibility complex (MHC) (Portaro et al., 1999; Silva et al., 1999; York et al., 2003). MHC bind to antigenic peptides derived from pathogens (Portaro et al., 1999; Silva et al., 1999; York et al., 2003). The binding of the peptides to MHC facilitates peptide display, which in the case of MHC Class I, involves the production of mature cytotoxic T lymphocytes. Cytosolic TOP targets antigenic peptides, thus limiting antigen presentation of MHC Class I (Portaro et al., 1999; Silva et al., 1999; York et al., 2003). Plants do not have an adaptive immune system. The function of TOPs in plant immunity and disease development is unclear.

In mammals, TOPs are redox-sensitive peptidases that form oligomers via intermolecular disulfide bonds (Sigman et al., 2003; Shrimpton et al., 1997). The disulfide bonds are mediated by multiple cysteine residues (Shrimpton et al., 1997; Shrimpton et al., 2003). The activity of the peptidase is regulated by oligomerization (Shrimpton et al., 1997; Shrimpton et al., 2003). The monomeric state constitutes the activate state of the enzyme (Shrimpton et al., 1997; Sigmam et al., 2003). The oligomeric states show reduced activity in comparison to the monomer (Demasi et al., 2008). In addition, the activities and oligomerization of mammalian TOPs are regulated by thiols (Demasi et al., 2008; Shrimpton et al., 1997). The enzymes are post-translationally modified by the thiol glutathione (Demasi et al., 2008; Malvezzi et al., 2012). Glutathione is the most abundant non-protein intracellular thiol, with tremendous importance in redox-regulation in plants (Dixon et al., 2005; Noctor et al., 2012). Glutathione is an antioxidant that is synthesized in the cytosol and is distributed in the chloroplasts and mitochondria (Noctor et al., 2012). During oxidative stress, glutathione interacts with proteins in a post-translational modification known as S-

glutathionylation (Dixon et al., 2005). S-glutathionylation is thought to protect proteins from the irreversible oxidation of cysteine residues (Dixon et al., 2005). Interestingly, S-glutathionylation of the monomer induces the highest active state of the mammalian TOPs (Demasi et al., 2008; Malvezzi et al., 2012). S-glutathionylation also facilitates the oligomerization of the enzymes, suggesting that mammalian TOP activity is tightly coordinated by glutathione levels and the cellular redox changes (Demasi et al., 2008; Malvezzi et al., 2012).

While mammalian TOP has been a subject of extensive research, the functions of TOPs in plants are not well understood. There are four TOP-like proteins in *A. thaliana*. TOP1 is an organellar metallopeptidase that is dually localized to the chloroplast and mitochondria (Kmiec et al., 2013; Moreau et al., 2013). The peptidase is capable of degrading transit peptides and presequences *in vitro* (Kmiec et al., 2013). It is postulated that the enzyme cleaves free-targeting peptides produced after protein transportation (Kmiec et al., 2013). Thus, TOP1 may prevent the accumulation of potentially toxic substrates produced during organellar transport (Kmiec et al., 2013). In addition, it is postulated that TOP1 is involved in the complete degradation of oxidized proteins (Kmiec et al., 2013). TOP2 is a cytosolic metallopeptidase (Kmiec et al., 2013; Moreau et al., 2013). It has been proposed that TOP2 cleaves peptides produced from the proteasome to limit the accumulation of substrates during oxidative stress (Polge et al., 2009). The activity of recombinant TOP1 and TOP2 is non-competitively inhibited by SA (Moreau et al., 2013). In plant extracts, SA impairs the degradation of synthetic TOP substrates (Moreau et al., 2013). TOP-like 1 (TOPL1) is an uncharacterized peptidase of unknown function. The protein is predicted to localize to the cytosol (Kmiec et al., 2013). The fourth TOP-like protein, TOPL2, is potentially dually localized to the mitochondria and chloroplasts (Carrie et al., 2015; Kleffmann et al., 2004). Past proteomic studies have found TOPL2 in the chloroplasts (Kleffmann

et al., 2004). Recent research has shown that TOPL2 may be potentially exported to the mitochondria (Carrie et al., 2015). Subcellular localization studies for TOPL2 have been inconclusive as TOPL2-GFP fusions are undetected (Carrie et al., 2015). Recently, evidence suggests that TOPL2 may act on mitochondrial presequences and transit peptides (Carrie et al., 2015). In a similar function to TOP1, TOPL2 may prevent the accumulation of free-targeting peptides produced during transport (Carrie et al., 2015). Thus, redundancy may exist between the organellar TOPs. The substrate specificity of TOPL2 suggests that the enzyme may target a wide range of peptides and may also have additional functions outside of transit peptide processing (Carrie et al., 2015).

Closing Remarks

The functions of TOP1 and TOP2 in the context of biotic stress and redox-related processes are unknown. Furthermore, the factors regulating TOP activity in plant systems are unclear. Our work aims to pioneer the understanding of TOPs in plant systems. This study characterizes the involvement of plant TOPs in plant immunity and oxidative stress. Furthermore, our work aims to explore the redox-regulation and thiol-sensitivity of TOP1 and TOP2. Also, we seek to identify candidate SABPs with potential roles in SA-regulated processes and plant immunity.

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CHAPTER 2

TOP1 AND TOP2 ARE THIOL-SENSITIVE PEPTIDASES THAT FORM REDOX-SENSITIVE OLIGOMERS

ABSTRACT

In this study, we investigated whether TOP1 and TOP2 are capable of forming redox-sensitive oligomers. We determined that TOP1 and TOP2 form homodimers. Additionally, TOP1 and TOP2 are capable of forming heterodimers. In addition, we provide evidence that TOP dimerization may be influenced by SA and redox reagents. Glutathione, a thiol-based redox-regulator, induces the oligomeric forms of TOP2. We propose that the oxidative oligomerization induced by glutathione occurs through a series of thiol-disulfide bond exchanges. Our work supports that TOPs self-associate via redox-sensitive bonds. Characterization of TOPs shows that the activity of the enzymes is thiol-sensitive. The thiol sensitivity of TOP1 and TOP2 is distinct from their mammalian counterpart. Lastly, we characterized the involvement of TOPs in plant oxidative stress responses.

INTRODUCTION

Salicylic acid (SA) is a critical signaling molecule that regulates an array of biotic and abiotic stress responses (Vicente and Plasencia, 2011; Vlot et al., 2009). Recent advancements in salicylic acid targets have discovered a plethora of proteins with SA-binding capacities. High-throughput screening using protein microarrays and mass spectrometry have identified over 150 salicylic acid binding proteins (SABPs) (Manohar et al., 2015; Moreau et al., 2013). The multitude of novel SABPs suggests that SA may exert its effects by modulating a wide range of proteins, rather than a few receptor-like proteins (Klessig et al., 2016). Interestingly, a wide range of SABPs discovered with both traditional approaches and high-throughput approaches have shown a theme of being related to redox and oxidative stress (Durner and Klessig, 1995; Manohar et al., 2015; Sanchez-Casas and Klessig, 1994; Vlot et al., 2009). It is thought that SA targets these redox-related enzymes to potentiate the redox changes induced by the hormone (Vlot et al., 2009). Protein microarray screenings have identified thimet oligopeptidase 1 (TOP1) as a SABP (Moreau et al., 2013). In addition, a TOP1 homolog, TOP2 was confirmed to bind to SA, albeit at a lower affinity (Moreau et al., 2013). Outside of plant systems, eukaryotic TOPs are firmly established as redox-regulated enzymes (Demasi et al., 2008; Malvezzi et al., 2012; Shrimpton et al., 1997). However, the redox-related processes of plant TOPs are unknown.

TOP1 contains a transit peptide which facilitates the dual localization of the enzyme to the chloroplasts and mitochondria (Kmiec et al., 2013; Moreau et al., 2013). Biochemical characterization of TOP1 activity suggests that the enzyme targets and degrades transit peptides in the organelles (Kmiec et al., 2013). In addition to TOP1's role in transit peptide degradation, the broad substrate specificity suggests that TOP1 functions in general organellar peptide degradation and in an array of proteolytic processes (Kmiec et al., 2013). A model of TOP1 activity suggests that the enzyme may also contribute to the complete degradation of oxidized proteins produced during oxidative stress (Kmiec et al., 2013). The predicted role of plant TOPs in oxidative stress also extends to TOP2. Cytosolic TOP2 is predicted to

function downstream of the proteasome and is thought to target and cleave peptides generated from oxidative stress (Polge et al., 2009). Recent characterizations found TOP1 and TOP2 to be involved in certain aspects of plant immunity, including resistance towards specific avirulent infections and cell death (Moreau et al., 2013). Furthermore, TOP1 and TOP2 activity is receptive to SA and is non-competitively inhibited by the hormone (Moreau et al., 2013). However, there is much unknown about regulation and function of plant TOPs.

While the regulation of plant TOP activity is unclear, mammalian TOP has multiple layers of regulation that dictate the enzyme's activity. Mammalian TOPs are cysteine-rich redox-sensitive peptidases (Malvezzi et al., 2012). The peptidases form redox-sensitive intermolecular disulfide bonds that influence the oligomeric state of the enzymes (Demasi et al., 2008; Malvezzi et al., 2012; Shrimpton et al., 1997). The monomeric form of the enzyme is active, while the oligomeric forms typically constitute the inactive state of the enzyme (Demasi et al., 2008; Shrimpton et al., 1997). Therefore, the activity is largely influenced by redox-regulated oligomerization. Cysteine residues are highly involved in eukaryotic TOP regulation (Sigman et al., 2003; Shrimpton et al., 1997). Interestingly, plant TOP1 and TOP2 are not cysteine-rich like their mammalian counterparts. Mature TOP1 has three cysteines and TOP2 has four cysteine residues. It remains to be seen whether redox-regulation of TOPs occurs in plant systems.

SA accumulation results in redox-changes that are vital to SA-signaling (Mou et al., 2003; Vlot et al., 2009). SA-induced redox changes drive the monomerization of the SA-signal regulator, NPR1 (Vlot et al., 2009). The redox-driven monomerization results in the shift towards the active form of NPR1 and is imperative for SA-signaling. However, the extent of this relationship and the mechanism that drives SA-induced redox changes is unclear. Studies suggest that the hormone induces changes in the accumulation of the major redox regulator molecule glutathione (Freeman et al., 2004; Li et al., 2013). Glutathione is the most prevalent non-protein cellular thiol. Glutathione is an essential antioxidant and redox regulator in plants. The metabolite has a plethora of functions in plants (Foyer and Nocter, 2011; Nocter et al., 2012). The

roles that glutathione play in plants includes regulation of reactive oxygen species (ROS) signaling, reactive nitrogen signaling (RON), oxidative stress responses, heavy metal detoxification, and plant immunity (Hiruma et al., 2013; Nocter et al., 2012). Interestingly, mammalian TOPs are thiol-sensitive (Demasi et al. 2008). Glutathione induces oxidative oligomerization of mammalian TOP (Demasi et al. 2008). Furthermore, the activity of mammalian TOPs is modulated by thiols, as incubation with thiols such as dithiothreitol (DTT) or glutathione may activate or inhibit the activity of the peptidase (Demasi et al., 2008; Sigman et al., 2003; Shrimpton et al., 1997). Whether plant TOPs are subject to the same form of thiol regulation is unclear.

Many forms of TOP regulation exist in other eukaryotic systems that are unknown to occur in plant systems. Our work aims to pioneer the study of redox-regulation in plant TOP1 and TOP2. We hypothesize that TOP1 and TOP2 self-associate via redox-sensitive intermolecular bonds. Analogous to mammalian TOP, we hypothesize that TOP1 and TOP2 are subject to similar thiol-based regulation. In this study, our findings identify novel aspects of TOP regulation in plants.

Our work suggests that TOP oligomerization is redox-sensitive. Furthermore, our study indicates that TOP1 and TOP2 are thiol-sensitive peptidases. Therefore, plant TOPs have similar forms of regulation to that of mammalian TOPs. However, our additional findings suggest there are still key differences that distinguish TOP1 and TOP2 from their other eukaryotic counterparts. Also, our work also characterized the involvement of TOPs in specific oxidative stress responses.

MATERIALS AND METHODS

Split Luciferase assay in plant protoplasts.

The coding sequence of *TOP1* and *TOP2* was amplified from universal cloning vector U10200/U10217. *TOP1* and *TOP2* were cloned into pENTR entry vector using BP clonase. *TOP1* and *TOP2* were then cloned into pDuEx-DC6 and pDuEx-AC6 luciferase-fusion destination vectors using LR clonase (Thermo Fischer Scientific). pDuEx-DC6 and pDuEx-AC6 destination vector constitute the N-terminal and C-terminal of the luciferase enzyme. HopF2 and Map Kinase Kinase (MKK5) luciferase fusion constructs were used as a positive control. Protein-interactions unite the two-

halves of the luciferase molecule which in turn restores luciferase activity. Plasmid extraction was performed utilizing Zymo's Zyppy Maxi Plasmid Kit and Qiagen Maxi Plasmid Kit (Zymo Technology and Qiagen). Zymo's Clean and Concentrator kits were used for additional plasmid purification and concentrating. A centrifugal evaporator was utilized for additional concentration.

Protoplast transformations and split-luciferase assays were performed using a modified protoplast extraction and polyethylene glycol transformation method derived from Singh et al., 2014, Wu et al., 2009, and Yoo et al., 2007. *Arabidopsis* leaves were detached from rosettes of short-day-grown plants (4-5 weeks) just before bolting. 3M Scotch Tape was applied to the leaves, and the epidermis was gently removed. After the layer had been removed, the leaves were subjected to cellulase and macerozyme digestion (Yakult Pharmaceuticals). Leaves were gently shaken every 20 minutes in digestion solution for 1-2 hours to slowly release protoplasts in the solution. After the abaxial side of the leaf became transparent, the digestion solution was extracted and filtered. Additional digestion solution was used to rinse any lingering protoplasts in the digested leaves. The solution was filtered and combined with the earlier extract. The filtrate was gently transferred into centrifuge tubes. W5 buffer was added to the top of the filtrate and the solution was centrifuged. The protoplast bands were extracted, washed, and then subsequently used for the transformation assay. Protoplasts were transferred into 96-well microwell plates and incubated with *TOP* or positive control fusion construct. Protoplasts were transformation with plasmids via polyethylene glycol transformation. After 12-16 hours of incubation in complete darkness, protoplasts were then incubated with ViviRen (Promega renilla luciferase substrate) in the presence or absence of SA in complete darkness. The well plate was immediately placed in BioTek Synergy Microwell Plate Reader where luminescence was read.

Protein Production

Coding sequences of *TOP1* and *TOP2* were amplified from universal cloning vector U10200/U10217. *TOP1* and *TOP2* were cloned into pENTR entry vectors using BP clonase. TOP1 and TOP2 were cloned into pET-28 and pET-32 HIS-tagged

destination vectors. The fusion constructs were transformed into the BL21 protein production strain. Bacteria were then grown to an optical density of 0 .8 and then subjected to Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. IPTG incubation was used for the induction of protein production for 12-24 hours at 15-20°C. Bacterial cells were then frozen and thawed repeatedly in protein-extraction buffer (Thermo-Scientific). The solution was then sonicated for 5-10 minutes on ice and centrifuged. Supernatant was collected. The pellet was then partially re-suspended in protein extraction buffer and re-centrifuged. The supernatant was pooled and subjected to purification. Protein purification was done via cobalt HIS-tagged purification followed by filtered size-based purification based and gel filtration. Protein extracts were incubated in His-Pur cobalt resin columns in 4°C and stirred using an orbital shaker. After a 30 minute incubation period, the resin was rinsed as per manufacturer instruction (Thermo-scientific). After cobalt HIS-tag purification, the purified extract were subjected to AMICON ULTRA 50K Centrifuge size-exclusion filters (EMD Millipore) for further purification and concentration to the extract. The solution was then subjected to size exclusion chromatography for an elution profile and additional purification. Eluted TOP proteins were then concentrated using AMICON Ultra filters and utilized for successive experiments. AMICON ULTRA 50K filters were used to concentrate the monomer and AMICON Ultra filters 150K MWCO filter was used to concentrate the dimer.

Yeast Two-Hybrid

The direct influence of SA was assessed by the yeast two-hybrid protocol as described by Fu et al., 2013. *TOP2* and *AS1* (asymmetric 1) were cloned into pGADT7 and pGBKT7 in *Saccharomyces cerevisiae.* The constructs were transformed into yeast strains AH109 and Y187 and used for the subsequent assays. Interaction assays were performed on SD-Trp-Leu-His selection media with 3-aminotriazole in the presence or absence of SA. Interaction assays are as follows; TOP2-pGBKT7/TOP2-pGADT7 and TOP-pGBKT7/AS1-pGADT7 (negative control).

Gel Filtration Chromatography

TOP1 and TOP2 recombinant proteins were incubated with reducing agent dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) to break and impair potential formation of disulfide bonds in TOP1 and TOP2. For glutathione, TOP1 and TOP2 recombinant protein was incubated with 500uM oxidized glutathione. After incubation, TOPs were then subjected to size-exclusion chromatography to remove any additive agent, collect the monomeric, dimer, multimeric fractions, and obtain an elution profile. TOP samples were injected into Superdex 200 gel filtration column (GE Life Sciences). Size-exclusion chromatography separated the oligomeric states of TOPs on the basis of size. Large molecules elute at lower retention time than smaller molecules.

Enzymatic Assay

Purified monomer and dimer fractions were concentrated using AMICON Ultra filters. The enzyme (0.1 μg) was incubated in the TRIS-HCl reaction buffer with 20uM MCA-Dnp substrate. The substrate utilized for the DTT assays, pH assays, and deletion mutant assays was Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys (Dnp)-OH fluorogenic substrate (Enzo Life Sciences). The substrate emits detectable fluorescence upon cleavage. Substrate cleavage was detected by the Synergy 4 microwell plate reader at excitation wavelength 328nm and emission wavelength 393nm.

For ATP Enzymatic Assay

0.01ug of protein was incubated in a 99uL reaction buffer (sodium phosphate buffer with 10uM fluorogenic substrate and 250uM ATP). The substrates utilized for the assay was matrix metalloproteinases (MMP) Nma–Dnp fluorogenic substrate. The reaction was monitored in a Tecan microwell plate reader. Fluorescence was detected at excitation 360nM and emission 465nM.

Site Directed Mutagenesis

QuikChange Lightning Site-Directed Mutagenesis Kit was utilized to mutate His-tagged TOP2 (Agilent). Primers for substrate site mutagenesis were developed by QuikChange Primer Design Program (Agilent). Primers for TOP2 deletion mutant

were F-CTGTAGTATCCAGCTGCGAAGCTACAGAGGAATC and R-GATTCCTCTGTAGCTTCGCAGCTGGATACTACAG. Site-directed mutagenesis was done according to manufacturer's protocol (Agilent). Protein was purified as previously described above.

Oxidative Stress Assays

Seeds were surface sterilized utilizing 25% bleach and .01% tween solution. After surface sterilization, the bleach solution was pipetted out and the seeds were rinsed with water. The seeds were sowed unto Murashige and Skoog agar or Cornell Osmocote Soil. Afterwards, the seeds were incubated in complete darkness at 4°C for 48 hours. Following the dark incubation, the plants were moved to growth chambers. The growth chamber temperature was set at 23 ± 2 °C. The plants were grown for 3 to 4 weeks and utilized for successive assays. The T-DNA insertion lines utilized for this experiments were *top1*-339, *top2*-127, and *top1*-339*top2*-127 (as previously described in Moreau et al., 2013).

For methyl viologen and SA assays, germination assays were done in petri dishes containing Murashige and Skoog agar. The Murashige and Skoog agar was supplemented with methyl viologen (MV) and/or SA. Seeds were germinated in Murashige and Skoog agar without MV or SA for the control. Seeds were stratified on Murashige and Skoog agar unless indicated otherwise. After stratification at 4°C in complete darkness, the seeds were transferred into 16-hour light and 8-hour dark condition. Germination was assessed by radicle emergence or green cotyledon emergence. To assess the effect of methyl viologen on mature rosette leaves, 3-4 week old plants were syringe infiltrated with 50μM MV. Photosystem efficiency was assessed by measuring Fv/Fm values with a fluorometer.

For continual light and cold stress assays, seeds were germinated and grown in 12-hour light and 12-hour darkness at (80-100 μmol m^{-2} s^{-1}) for 10 days. Afterward, the plants were transferred into continual light (250-300 μmol m^{-2} s^{-1}) and cold (8°C). Photosystem efficiency was assessed by measuring Fv/Fm values with a fluorometer. For high light assays, plants were grown in 150-200 μmol m^{-2} s^{-1} for 3 weeks and then transferred to high light conditions (800-900 μmol m^{-2} s^{-1}). The anthocyanin

accumulation and chlorophyll content were assessed. For anthocyanin content, the optical density of the anthocyanin extracts was read at 520 nm and 657 nm. Leaf tissue extracted from control or treated (high light) plants were homogenized in 1 ml acetone solution. The mixture was incubated at 4 °C and in complete darkness. The solution was extracted and the optical density was read at 663 nm and 646 nm (chlorophyll *a* and *b*) using a spectrophotometer.

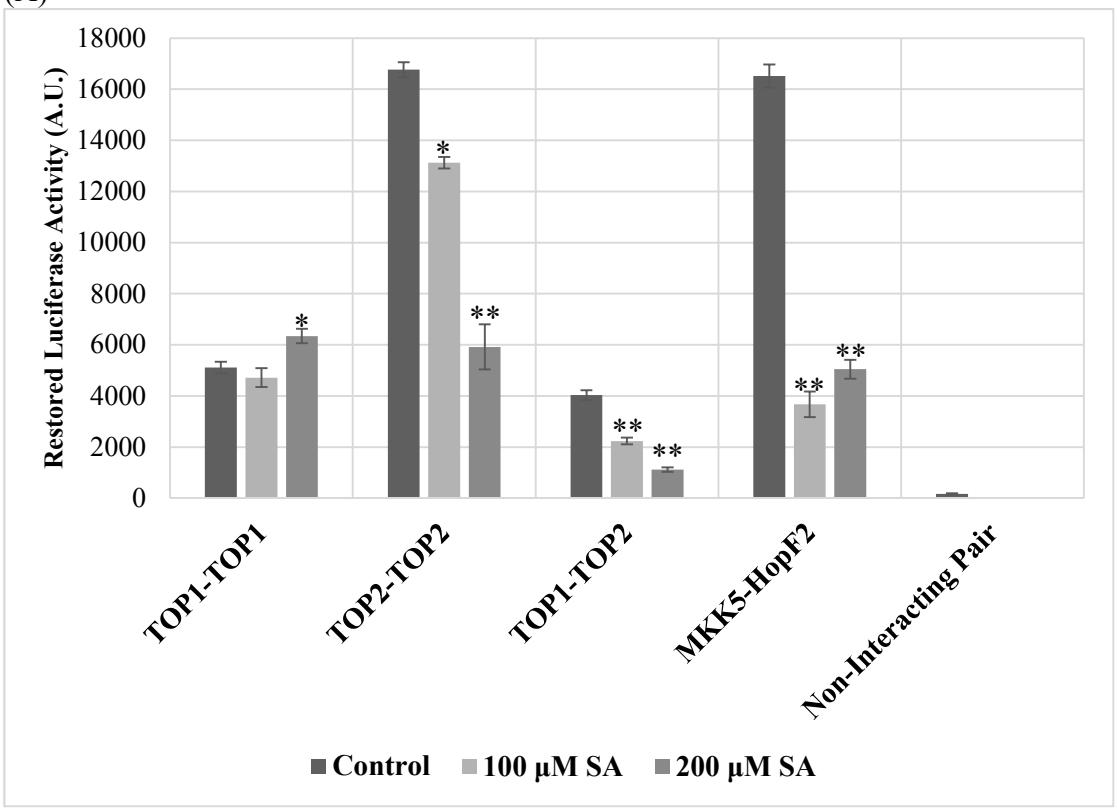
RESULTS

TOP1 and TOP2 form hetero- and homo-dimers

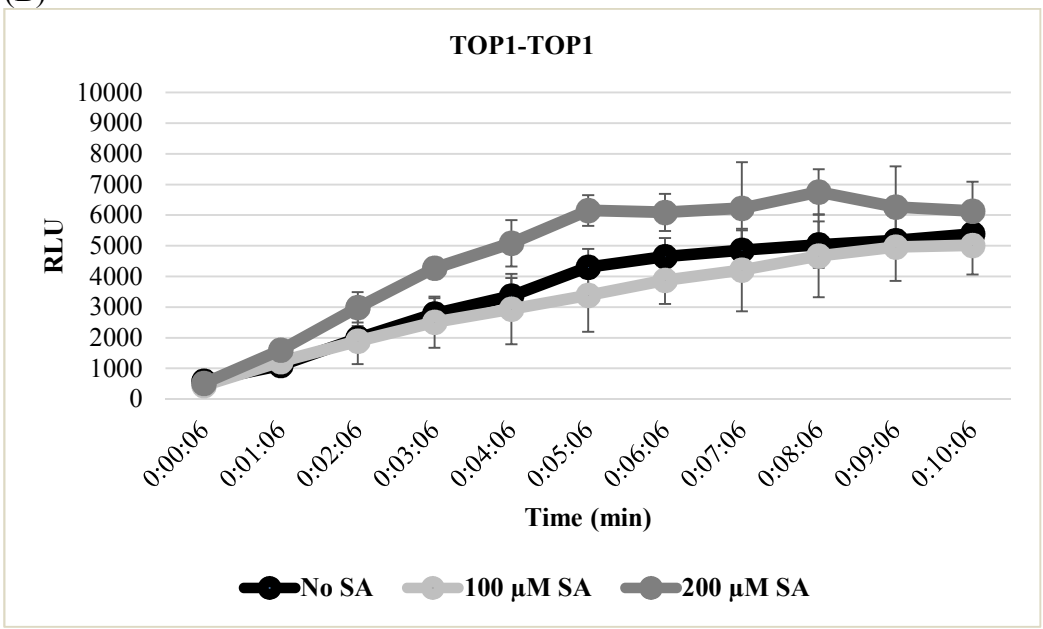
To explore whether plant TOPs are capable of self-associating, the dimerization of TOP1 and TOP2 was examined via the split-luciferase complementation assay (SLCA) in a plant protoplasts system. The assay allowed the dimerization of TOP1 and TOP2 to be assessed in the context of the cellular environment (Fujikawa and Kato, 2007). The technique involved the fusion of the N-terminal and C-terminal halves of the luciferase molecule (N-luciferase (Nluc) and C-luciferase (Cluc)) to TOP1 and TOP2. Protein-protein interactions would unite the halves of the luciferase molecule and in turn restore the enzymatic activity of the luciferase protein. MAP Kinase Kinase 5 (MKK5) and effector HopF2 interactions constituted the positive control for the assays (Wang et al., 2010) while non-interacting protein pairs (TOP1-Cluc fusions and MKK5-Nluc) were utilized as the negative control. We determined that *Arabidopsis* protoplasts expressing TOP1-Nluc and TOP1-Cluc restored luciferase activity, indicating that TOP1 self-associates (Figure 2-1 A, B). TOP1-NLuc and TOP1-Cluc exhibited higher luminescence than the non-interacting pair (Figure 2-1 A). Protoplasts expressing TOP2-Nluc and TOP2-Cluc pair showed higher detectable luminescence and greater luciferase activity than the TOP1-TOP1 pair and the non-interacting control (Figure 2-1 A-C). Furthermore, TOP1-Cluc and TOP2-Nluc interactions showed comparable luminescence intensity to that of protoplasts expressing TOP1-Cluc and TOP1-Nluc (Figure 2-1 A, B, D). Overall, our results support that TOP1 and TOP2 have the capacity to form homodimers and heterodimers.

Figure 2-1

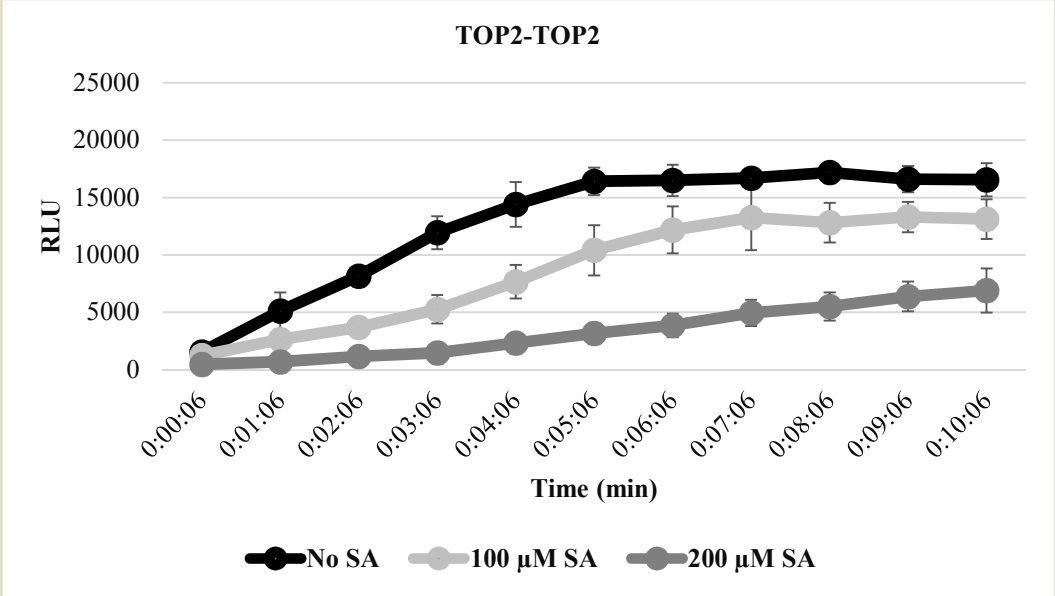
(A)


Restored Luciferase Activity (A.U.)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
**
**
**
**
TOP1-TOP1
TOP2-TOP2
TOP1-TOP2
MKK5-HopF2
Non-Interacting Pair
Control
100 µM SA
200 µM SA

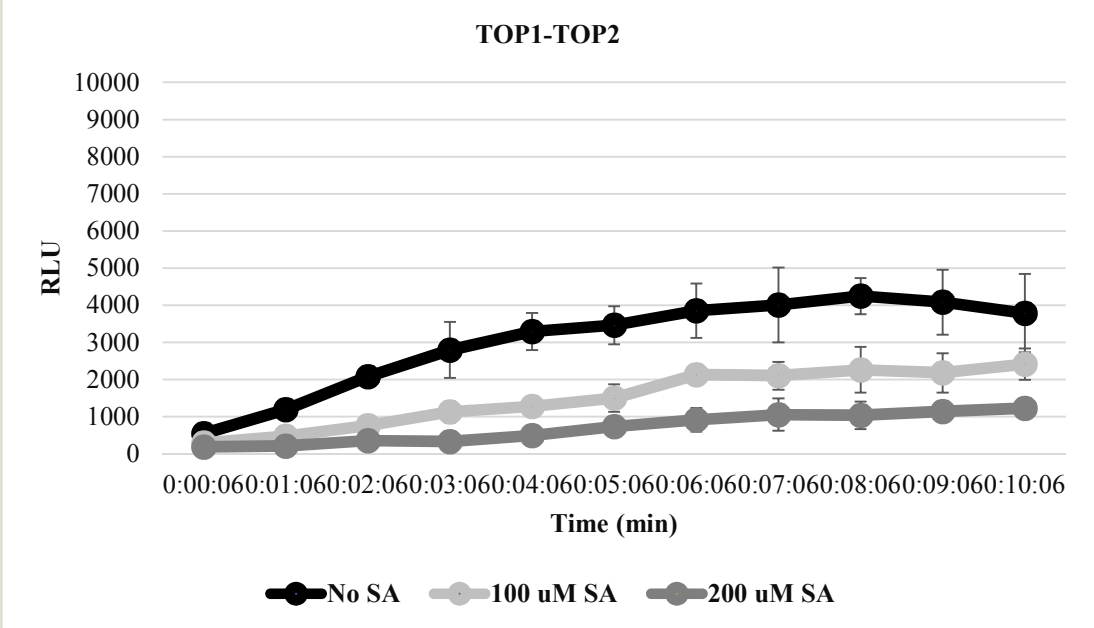
(B)


TOP1-TOP1
RLU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0:00:06
0:01:06
0:02:06
0:03:06
0:04:06
0:05:06
0:06:06
0:07:06
0:08:06
0:09:06
0:10:06
Time (min)
No SA
100 µM SA
200 µM SA

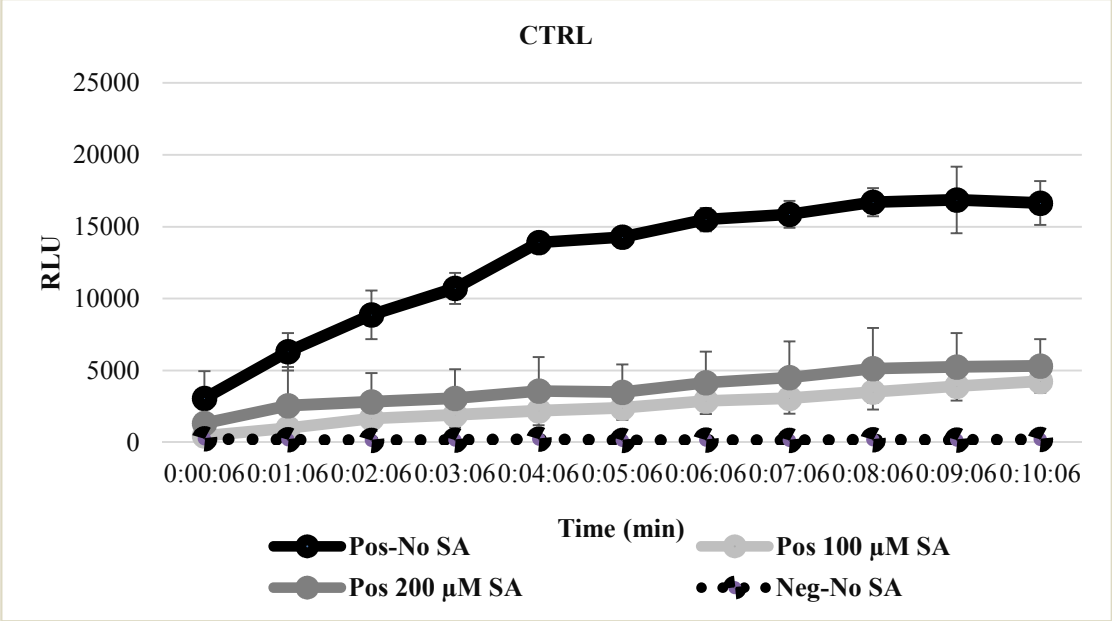
(C)


TOP2-TOP2
25000
20000
15000
10000
5000
0
RLU
0:00:06
0:01:06
0:02:06
0:03:06
0:04:06
0:05:06
0:06:06
0:07:06
0:08:06
0:09:06
0:10:06
Time (min)
No SA
100 µM SA
200 µM SA

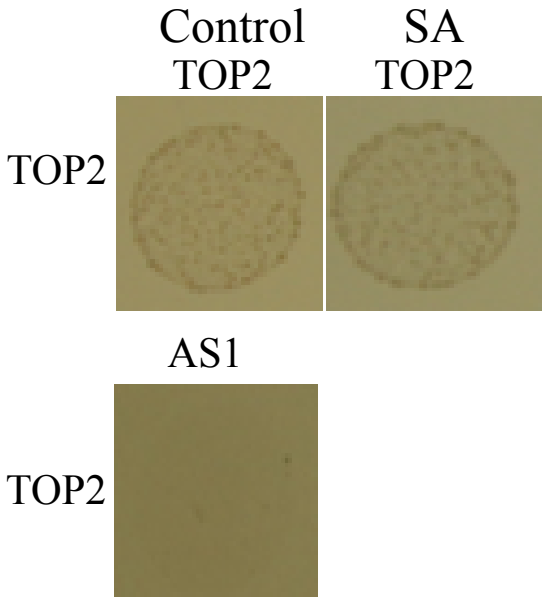
(D)


TOP1-TOP2
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
RLU
0:00:060:01:060:02:060:03:060:04:060:05:060:06:060:07:060:08:060:09:060:10:06
Time (min)
No SA
100 uM SA
200 uM SA

(E)



(F)



TOP1 and TOP2 form homodimers and heterodimers.

(A) Split-luciferase assays were performed in *Arabidopsis thaliana* protoplasts in the presence or absence of SA. Restored luciferase activity was used to detect interactions and bindings of interacting pairs. The luciferase intensity values, measured in the 07:06 and 10:06 min time interval after adding the luciferase substrate, were used to calculate the restored luciferase activity resulting from the

interactions (A.U. are arbitrary units). TOP1 is capable of forming homodimers. TOP2 is capable of self-associating and forming homodimers. TOP1 and TOP2 form heterodimers. SA modulates the interactions of the interacting pairs. Asterisks represent statistical significance between untreated (No SA) and treated (SA) interactions (Student's T-test) (*$p < 0.05$ and **$p < 0.01$) calculated from 3 replicates per protein pair tested.

(B) The relative luminescence of TOP1-TOP1 interactions over time in the presence or absence of SA. Relative luminescence units (RFU) emitted was used to assess interactions over time.

(C) The relative luminescence of TOP2-TOP2 interactions over time in the presence or absence of SA. RFU emitted was used to assess interactions over time.

(D) The relative luminescence of control interacting partners (MKK5 and HopF2) in the presence or absence of SA. RFU emitted was used to assess interactions over time.

(E) The relative luminescence of control interacting partners (MKK5 and HopF2) in the presence or absence of SA. RFU emitted was used to assess interactions over time.

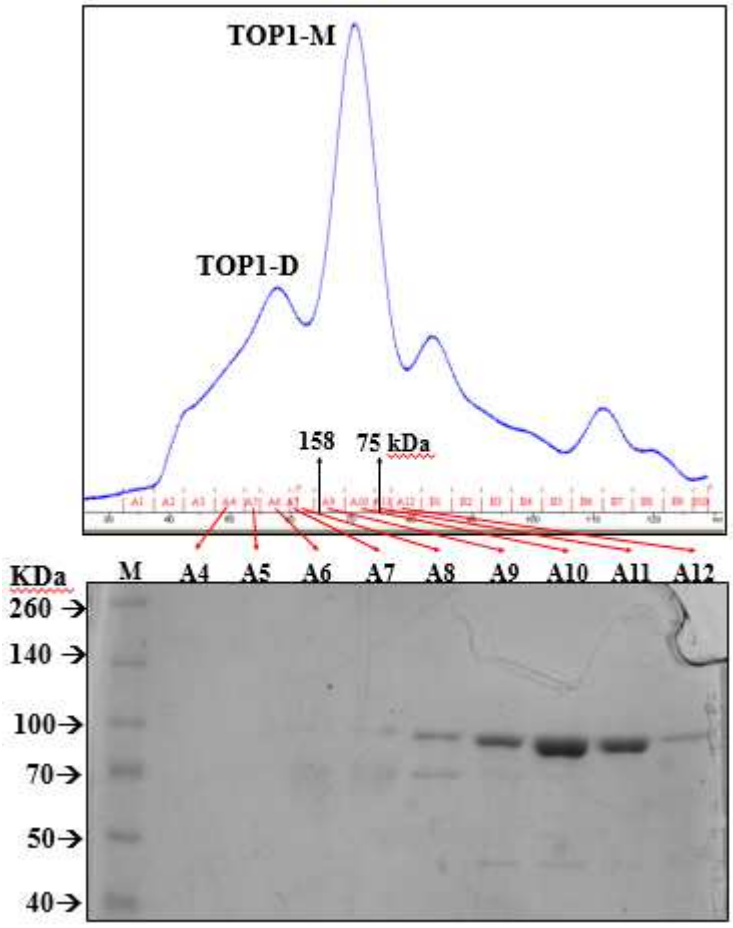
(F) Yeast two-hybrid assays were performed in the presence or absence of SA. TOP2 forms homodimers. 100 μM SA does not impair TOP2 homodimer formation. Non-interacting pair was used as the negative control.

To elucidate factors governing TOP dimerization, we utilized size exclusion chromatography to separate the dimeric and monomeric forms of TOP based on molecular weight. TOP1 and TOP2 elution profiles indicate that TOPs elute at two separate size fractions. TOP1 and TOP2 were detected in the specific eluted fractions of the gel filtration profile corresponding to the major peaks (Figures 2-2 A, B). At the shorter-retention peak, both TOP1-His and TOP2-His eluted at a molecular weight corresponding with approximately twice their molecular weight (Figures 2-2 A, B). Furthermore, the longer retention fraction peak at which TOP1-His and TOP2-His eluted correlate with the enzymes' actual MWs (Figures 2-2 A, B). Our findings suggest that the two eluted peaks are the dimeric form and monomeric forms of the two enzymes. The gel filtration profiles of TOPs suggest that in the utilized experimental parameters of the assays, the monomeric forms of TOPs were favored over that of the dimeric form.

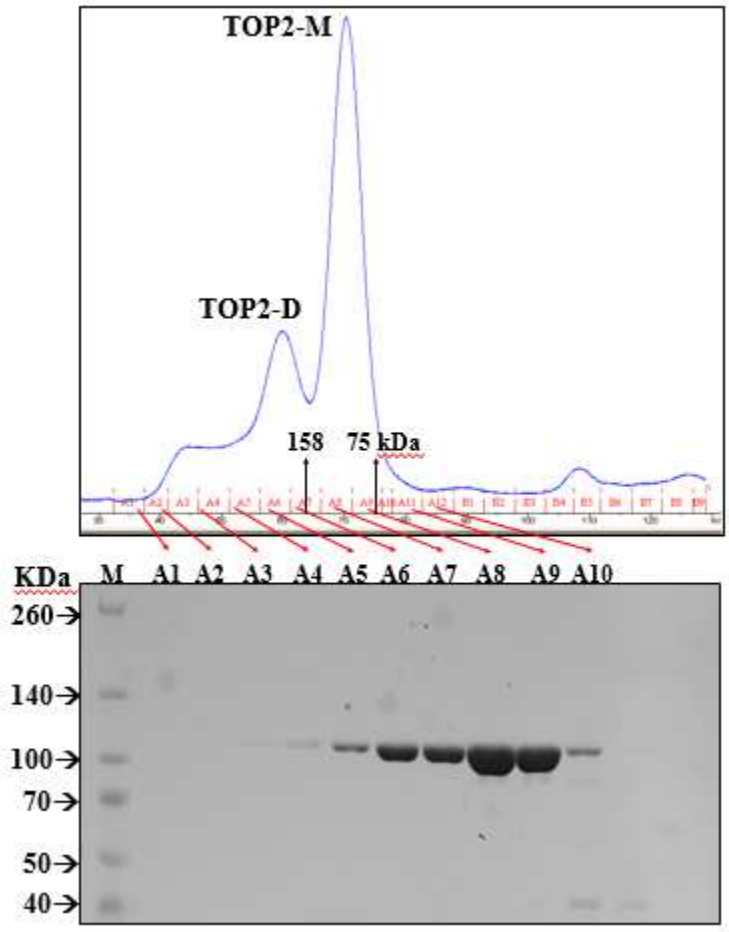
The influence of SA on TOP dimerization

To investigate the effects of SA on TOP dimerization, protoplasts expressing various pairs of TOP-luciferase fusions were incubated with 100 or 200 μM SA and restoration of luciferase activity was measured. SA treatment significantly lowered the intensity of the reconstituted luciferase in the case of TOP2-TOP2 and TOP1-TOP2 interactions compared to the no-SA condition (Figure 2-1 A, B, D). 100 μM SA reduced luciferase intensity of TOP2-luc dimers by approximately 30% and of TOP1-TOP2 dimers by 50%, while 200 μM SA reduced it by 75 and 80%, respectively (Figure 2-1 A, B, D). On the other hand, SA did not affect the TOP1-TOP1 dimerization interaction as much as the TOP2-TOP2 and TOP1-TOP2 interactions (Figure 2-1 A, B, C, D). Interestingly, SA did not significantly impair TOP1-TOP1 interactions at the tested concentrations. TOP1-TOP1 interactions appear to be insensitive (at 100 μM SA) or potentially increased by SA at 200 uM (Figure 2-1 A, C). We can't preclude the possibility that the null SA sensitivity of the TOP1 dimer in this system is a result of its localization in chloroplasts and mitochondria. The

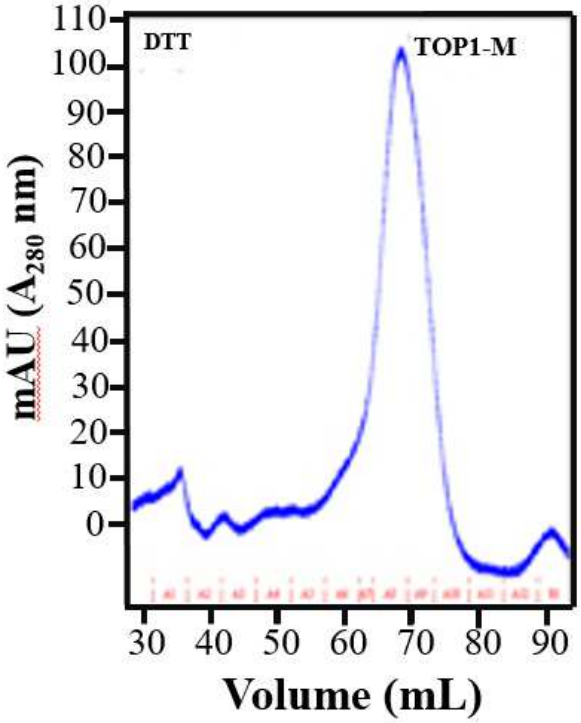
Figure 2-2
(A)


TOP1-M
TOP1-D
158
75 kDa
KDa
M
A4
A5
A6
A7
A8
A9
A10
A11
A12
260
140
100
70
50
40

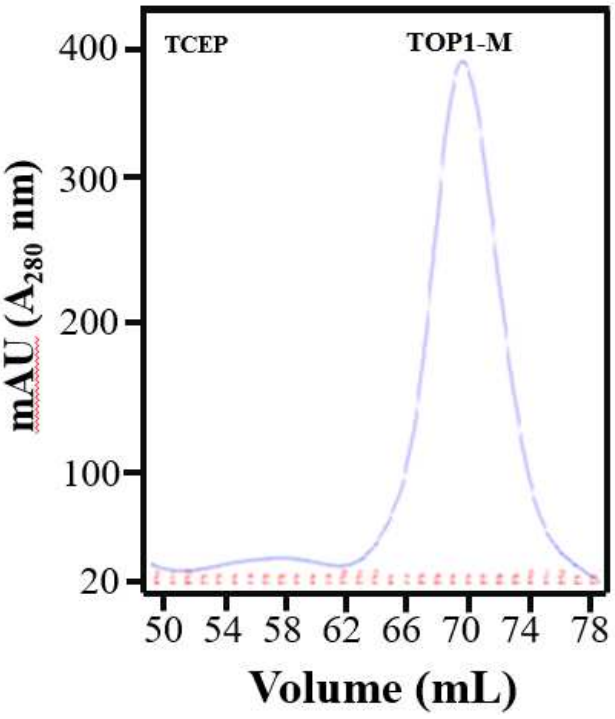
(B)


TOP2-M
TOP2-D
158
75 kDa
KDa
M
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
260
140
100
70
50
40

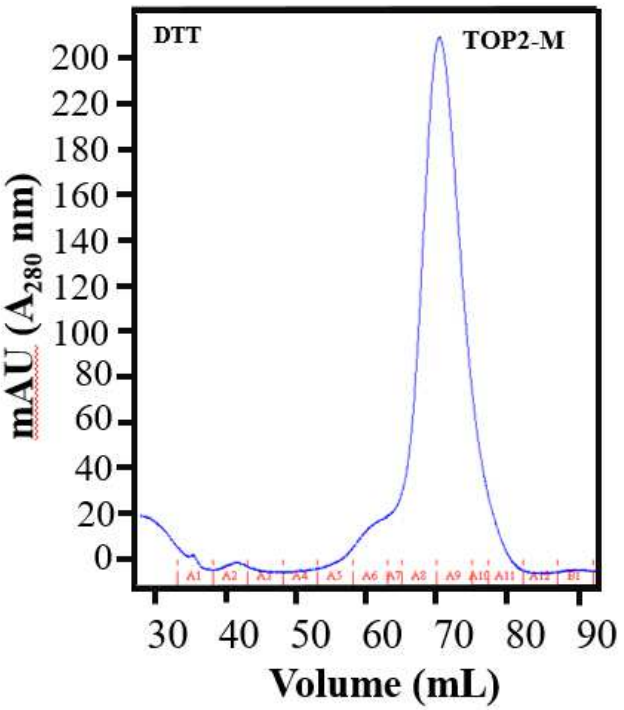
(C)


DTT
TOP1-M
mAU (A_{280} nm)
110
100
90
80
70
60
50
40
30
20
10
0
30 40 50 60 70 80 90
Volume (mL)

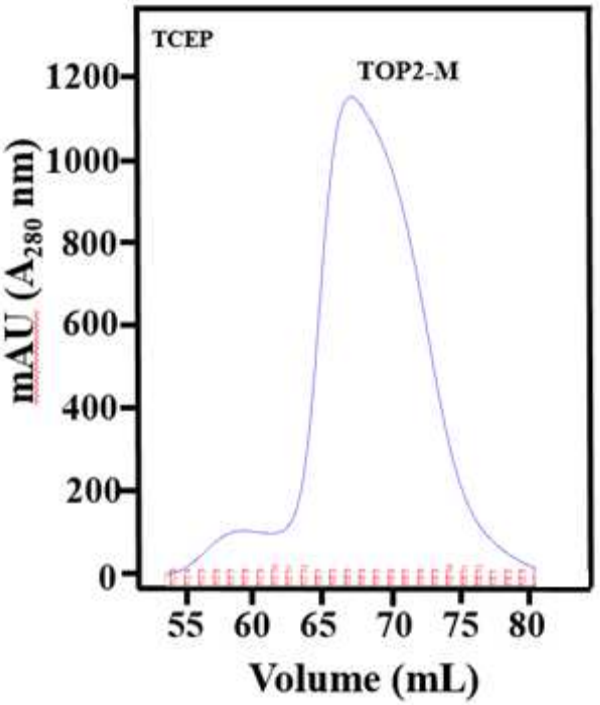
(D)


TCEP
TOP1-M
mAU (A_{280} nm)
400
300
200
100
20
50 54 58 62 66 70 74 78
Volume (mL)

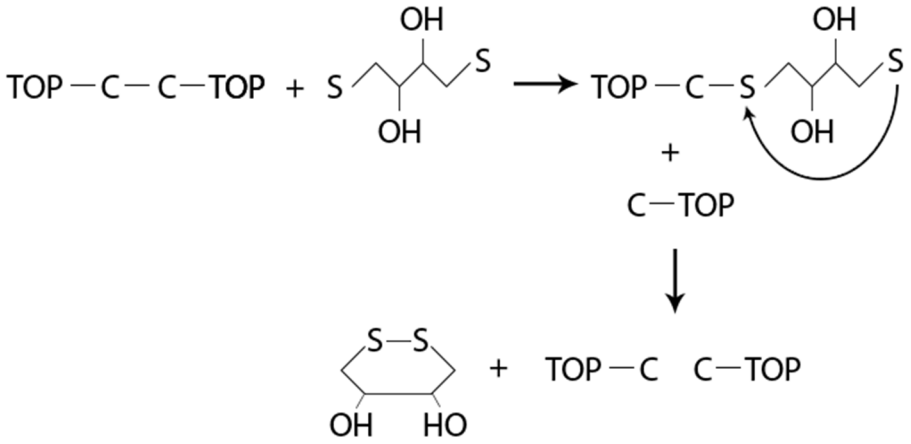
(E)


DTT
TOP2-M
mAU (A280 nm)
200
220
180
160
140
120
100
80
60
40
20
0
30
40
50
60
70
80
90
Volume (mL)

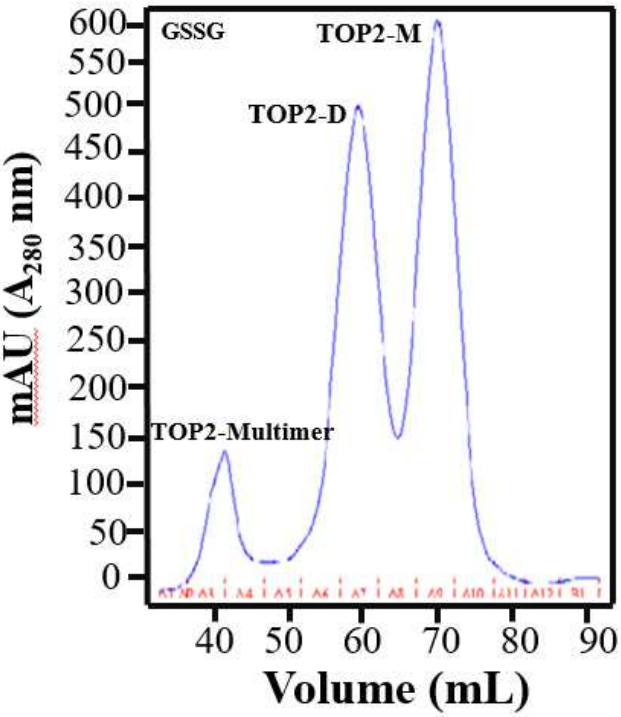
(F)


TCEP
TOP2-M
mAU (A280 nm)
1200
1000
800
600
400
200
0
55
60
65
70
75
80
Volume (mL)

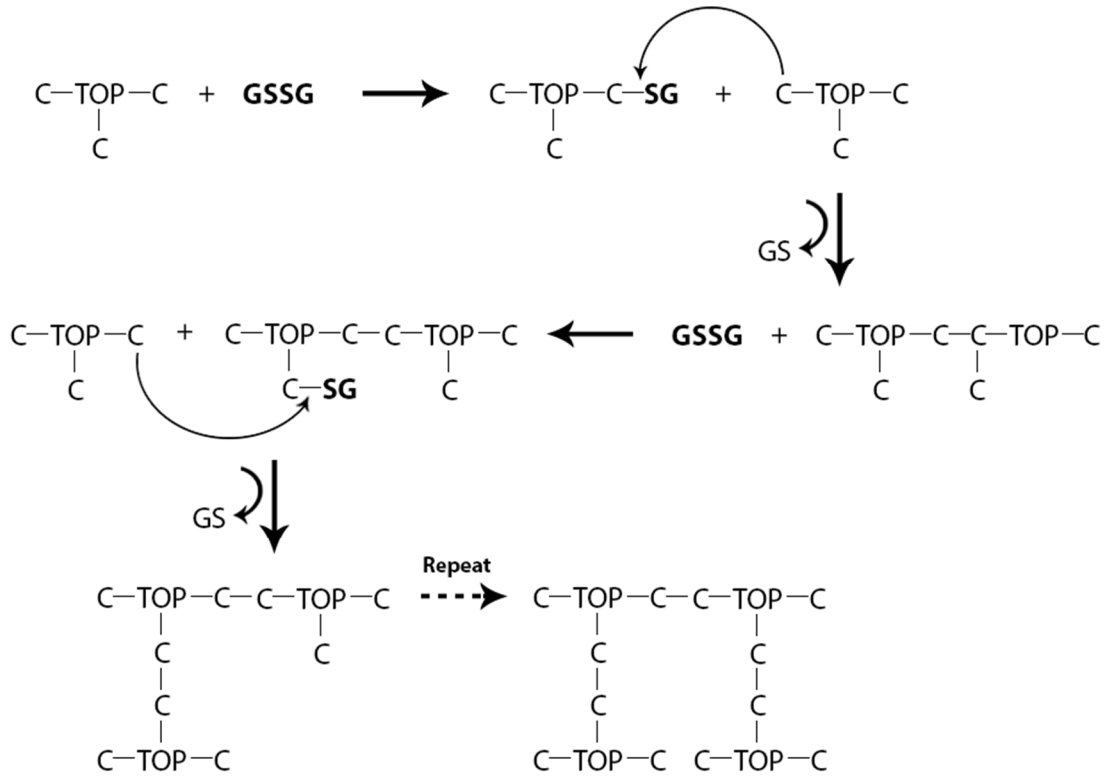
(G)


TOP—C—C—TOP
OH
S
OH
TOP—C—S
OH
S
OH
+
C—TOP
S—S
OH
HO
+
TOP—C
C—TOP

(H)


GSSG
TOP2-M
TOP2-D
TOP2-Multimer
mAU (A280 nm)
600
550
500
450
400
350
300
250
200
150
100
50
0
40
50
60
70
80
90
Volume (mL)

(I)



The oligomers of TOP1 and TOP2 are redox-sensitive.

(A) TOP1 obtained from cobalt His-tagged purification was subjected to size-exclusion chromatography. The elution profile in tandem with SDS page of the fractions indicates that TOP1 elutes primarily at two different size fractions corresponding to the sizes of the monomer and dimer.

(B) TOP2 obtained from cobalt His-tagged purification was subjected to size-exclusion chromatography. The elution profile in tandem with SDS page of the fractions indicates that TOP2 elutes primarily at two different size fractions corresponding to the sizes of the monomer and dimer.

(C) The elution profile of TOP1 in the absence or presence of reducing agent. TOP1 was incubated with 500 μM dithiothreitol. Incubation with the redox reagents results in the elution of peak corresponding with the size of monomeric TOP1.

(D) The elution profile of TOP1 in the absence or presence of 1 mM Tris-(2-carboxyethyl)phosphine (TCEP). Incubation with the redox reagents results in the elution of peak corresponding with the size of monomeric TOP1.

(E) The elution profile of TOP2 in the absence or presence of redox agents. TOP2 was incubation with 500 μM dithiothreitol. Incubation with reducing agents results in predominantly one peak corresponding to the size of monomeric TOP2.

(F) The elution profile of TOP2 in the absence or presence of 1mM TCEP. Incubation with reducing agents results in one peak corresponding to the size of monomeric TOP2.
(G) Working model of dithiothreitol reduction of TOP1 and TOP2 dimer. A thiol group of dithiothreitol interacts with a cysteine residue (C) of TOP resulting in the displacement of the disulfide bond between two TOP molecules. The free thiol group of dithiothreitol interacts with the other thiol of DTT.
(H) Incubation with oxidized glutathione results in three peaks corresponding to the monomeric, dimeric, and oligomeric form of TOP2.
(I) Working model of TOP2 oxidative oligomerization. Oligomerization of TOP2 is induced by oxidative glutathione (GSSG). Oxidized glutathione interacts with the cysteine residues (C) of TOP2 to form a TOP-glutathione bound intermediate. In a thiol-exchange reaction, another TOP molecule displaces the bound glutathione and forms the oligomeric form. The reactions may proceed in the presence of glutathione to induce the higher forms of TOP2.

amount of the exogenously applied SA that may be transported into the organelles within our experimental timeframe is unknown; it may be that SA does not accumulate to a threshold high enough to elicit a dramatic effect on TOP1 dimerization. The intensity of protoplasts expressing the positive interaction MKK5-HopF2 pair was decreased to similar levels in the presence of 100 or 200 μM SA (Figure 2-1 A, E). In all, our results indicate that TOP1 *in vivo* dimerization is more resistant to exogenous SA than TOP2 *in vivo* dimerization.

To elucidate the direct effects of SA on TOP self-associations, we performed Y2H assays to assess TOP2 interactions in the presence and absence of exogenous SA. The Y2H assay has been established as a method for assessing the direct effects of SA on protein-protein interactions (Fu et al., 2013). In tests utilizing TOP2 as bait and prey, yeast showed growth in the selective media (Figure 2-1 F). The results confirm TOP2's ability to form homodimers. There was no effect of exogenous SA on TOP dimerization (Figure 2-1 F). Direct SA did not alter TOP2-TOP2 interactions in Y2H. The results suggest that any potential influence of SA on TOP2 dimerization is not the result of direct SA binding.

TOP1 and TOP2 form redox-sensitive intermolecular disulfide bonds

SA induces redox-changes, which alter the binding capacity of SABPs such as NPR1 (Vlot el al., 2009). It is plausible that the dimerization of TOPs is regulated by redox changes analogous to mammalian systems. We hypothesize that TOP1 and TOP2 form redox-sensitive intermolecular bonds. To test this hypothesis, we examined TOP-TOP interactions in the presence of redox-modulating molecules. Dithiothreitol (DTT) is a thiol-based reducing agent used to break disulfide bonds and is capable of affecting the activity of many redox-sensitive proteins (Cleland, 1964). DTT incubation results in a reduction of redox-sensitive bonds of mammalian TOP. Recombinant TOP1 and TOP2 were incubated with 500 μM DTT and passed through the gel filtration column. TOPs' elution profiles showed a dramatic shift toward the monomeric fraction with the peak corresponding to the dimer being eliminated almost entirely after incubation with the thiol-based reductant (Figure 2-2 C, E). To confirm

that TOP1 and TOP2 bonds are susceptible to reduction, the proteins were incubated with 1mM TCEP. TCEP is a reducing agent that is utilized to break disulfide bonds (Burns et al., 1991). As with DTT, TCEP incubation resulted in the diminishing dimer fractions and thus the elution of predominantly monomeric size fractions in both TOP1 and TOP2 (Figure 2-2 D, F). We propose a model in which DTT breaks the disulfide bond between two TOP molecules in Figure 2-2 G. A thiol group of DTT interacts with a cysteine residue of TOP which displaces the preexisting disulfide bond forming a TOP-DTT intermediate (Figure 2-2 G). The second thiol group of DTT interacts with the other thiol group of DTT breaking the TOP-DTT intermediate. The result of the reaction are two TOP monomers (Figure 2-2 G).

Glutathione is a major intracellular thiol-based redox regulator in plants. To determine whether the formation of TOP oligomers is sensitive to thiols, recombinant TOP was incubated with oxidized glutathione. 500 μM of oxidized glutathione was incubated with TOP2. After incubation, TOP2 was subjected to gel filtration. The elution profile showed increase formation of the dimeric form of TOP2 (Figure 2-2 H). Furthermore, glutathione incubation resulted in the elution of a larger fraction, whose size corresponded to the multimeric form of TOP2. These findings suggest that TOP2 interactions are directly modulated by major cellular redox-regulators and thiol-based molecules. Furthermore, glutathione induces the oligomeric form of TOP2.

The direct effect of glutathione on TOP complex formation suggests that the oligomerization of TOP2 induced by glutathione occurs via multiple thiol–disulfide exchanges. We propose a mechanism for TOP oxidative oligomerization in Figure 2-2 I analogous to mammalian TOP (Demasi et al., 2008). A cysteine residue of TOP2 interacts with an oxidized glutathione molecule in a process known as S-glutathionylation (Figure 2-2 I). The S-glutathionylated residue is a result of a cysteine residue forming a disulfide bond directly with a glutathione molecule (Figure 2-2 I). The disulfide bond between the glutathione molecule and the cysteine residues is then attacked by a free cysteine residue of another TOP, forming a dimer. The dimer is a result of a thiol-disulfide exchange which displaced the previously bound glutathione and exchanges the former disulfide bond with a disulfide bond between two TOP

proteins (Figure 2-2 I). The reaction proceeds in the presence of oxidized glutathione. Glutathione interacts with another free cysteine residue in the TOP dimer and a disulfide bond is formed. A free cysteine residue of another TOP attacks the disulfide bond between glutathione and TOP. The disulfide bond between the TOP dimer and glutathione is exchanged, and another TOP becomes bound to the TOP dimer. The reaction is repeated with another TOP molecule to form a higher oligomeric state of the enzyme (Figure 2-2 I).

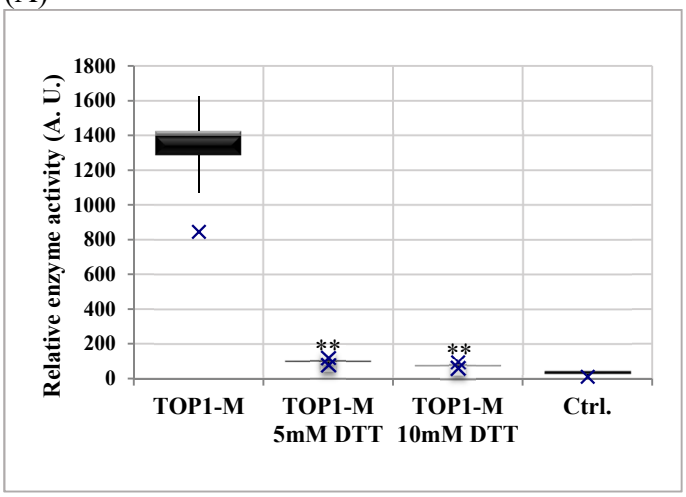
TOP1 and TOP2 activity is sensitive to thiols

TOP complex formation is sensitive to thiols such as DTT and glutathione. However, the influence of thiols on TOP activity is unknown. In mammalian systems, thiols modulate the activity of TOPs (Demasi et al., 2008; Shrimpton et al., 1997). At DTT concentrations greater than 1mM, mammalian TOP is inhibited by DTT, and at DTT concentrations <1mM, mammalian TOPs are activated (Lew et al., 1995; Orlowski et al., 1983; Shrimpton et al., 1997). We hypothesized that similar to that of mammalian TOP, TOP1 and TOP2 are sensitive to thiols. To test the hypothesis that plant TOPs are thiol-sensitive, the activity of recombinant TOP1 and TOP2 monomers and dimers was tested on a fluorogenic substrate in the absence (control) or the presence of concentrations of DTT. We incubated recombinant TOP1 and TOP2 with 5mM and 10mM DTT. 5mM and 10mM concentrations of DTT drastically inhibited the activity of TOP1 and TOP2 (Figure 2-3 A, D).

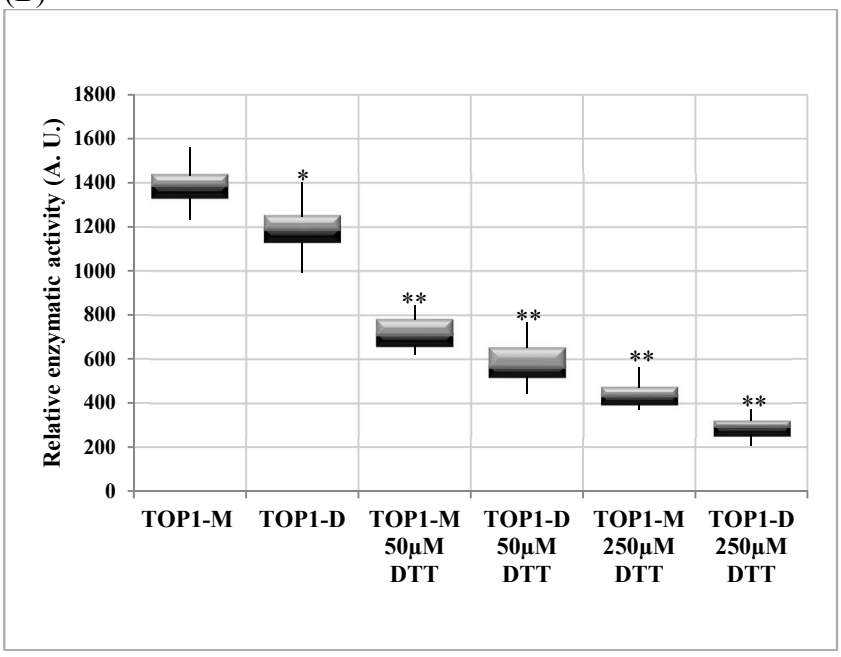
TOP1 dimeric fractions and monomeric fractions under control conditions reached the same level of activity after 10 min (Figure 2-3 C). However, their specific activities may differ over the duration of the experiment, with the higher form showing potential signs of lower activity values than the monomers (Figure 2-3 B). TOP2 monomers and dimers exhibited similar levels of activity under both control and reductive conditions (Figure 2-3 E). TOP1 monomeric and dimeric fractions were inhibited by the thiol-based reductant at 50 μM and 250 μM DTT (Figure 2-3 B, C). TOP2 monomeric and dimeric fractions were inhibited by the thiol-based reductant at 250 μM DTT (Figure 2-3 B, C). The sensitivities to thiols are distinct from that of mammalian systems in that DTT is inhibitory at concentrations lower than 1mM.

Figure 2-3

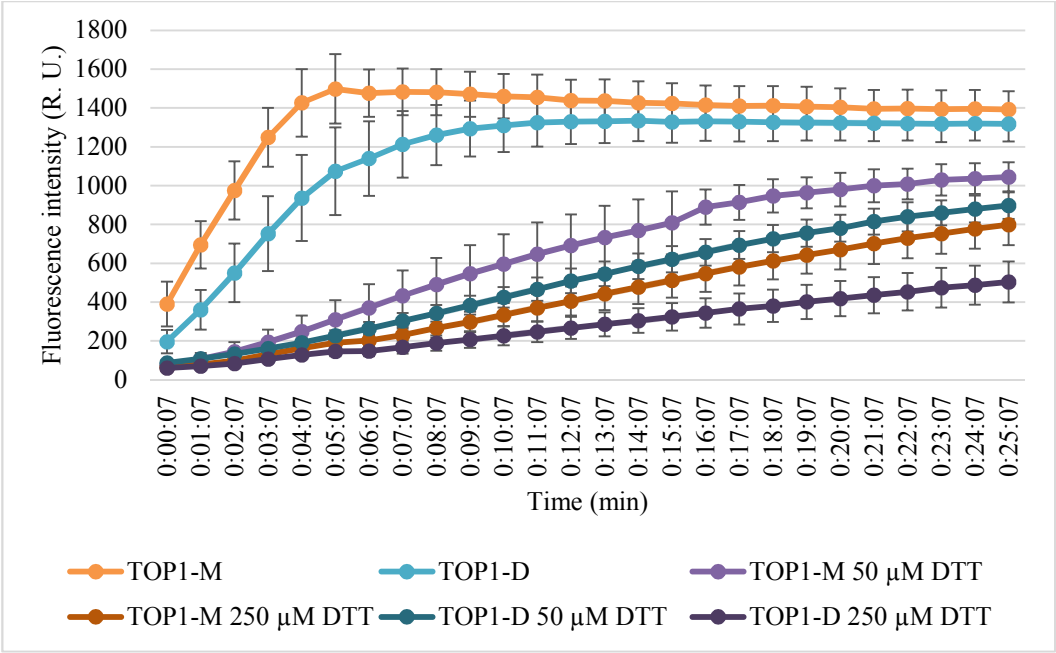
(A)


Relative enzyme activity (A. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
TOP1-M
TOP1-M 5mM DTT
TOP1-M 10mM DTT
Ctrl.

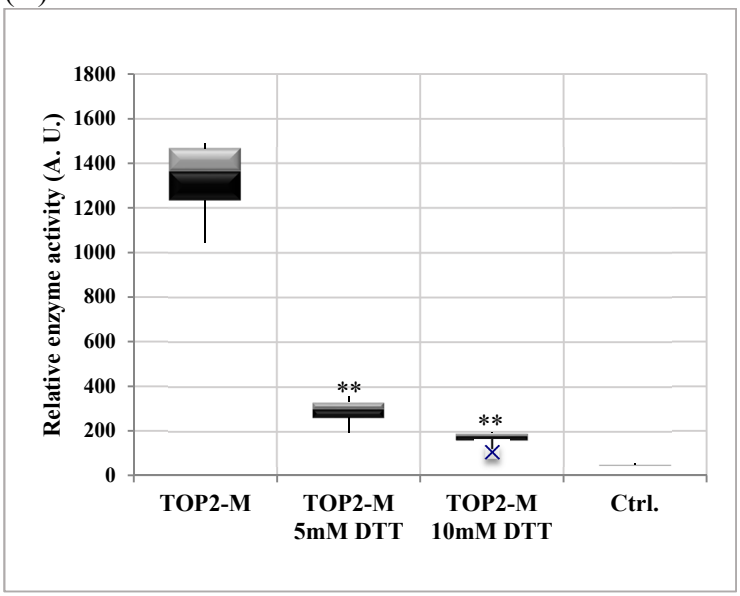
(B)


Relative enzymatic activity (A. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
*
**
**
**
**
TOP1-M
TOP1-D
TOP1-M 50μM DTT
TOP1-D 50μM DTT
TOP1-M 250μM DTT
TOP1-D 250μM DTT

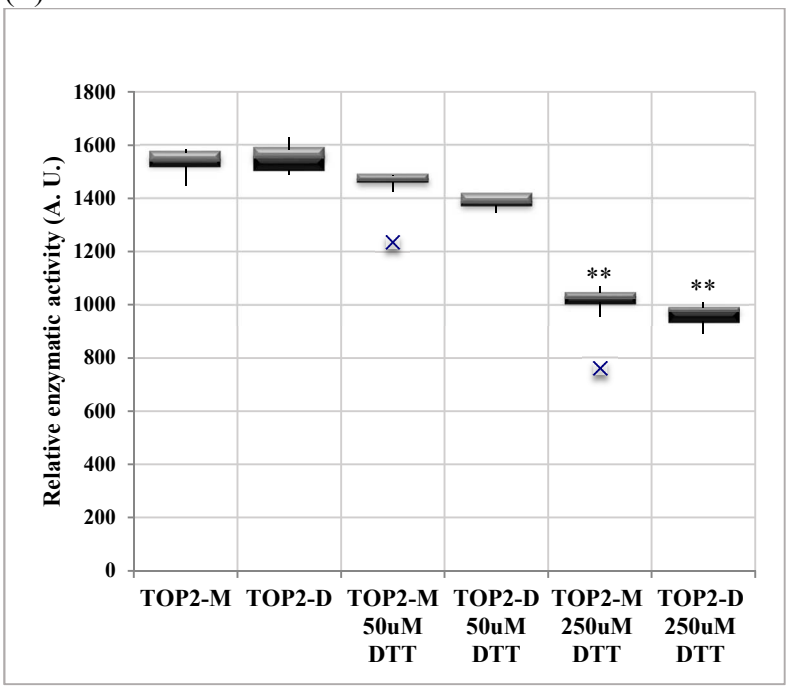
(C)


Fluorescence intensity (R. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
0:00:07
0:01:07
0:02:07
0:03:07
0:04:07
0:05:07
0:06:07
0:07:07
0:08:07
0:09:07
0:10:07
0:11:07
0:12:07
0:13:07
0:14:07
0:15:07
0:16:07
0:17:07
0:18:07
0:19:07
0:20:07
0:21:07
0:22:07
0:23:07
0:24:07
0:25:07
Time (min)
TOP1-M
TOP1-D
TOP1-M 50 µM DTT
TOP1-M 250 µM DTT
TOP1-D 50 µM DTT
TOP1-D 250 µM DTT

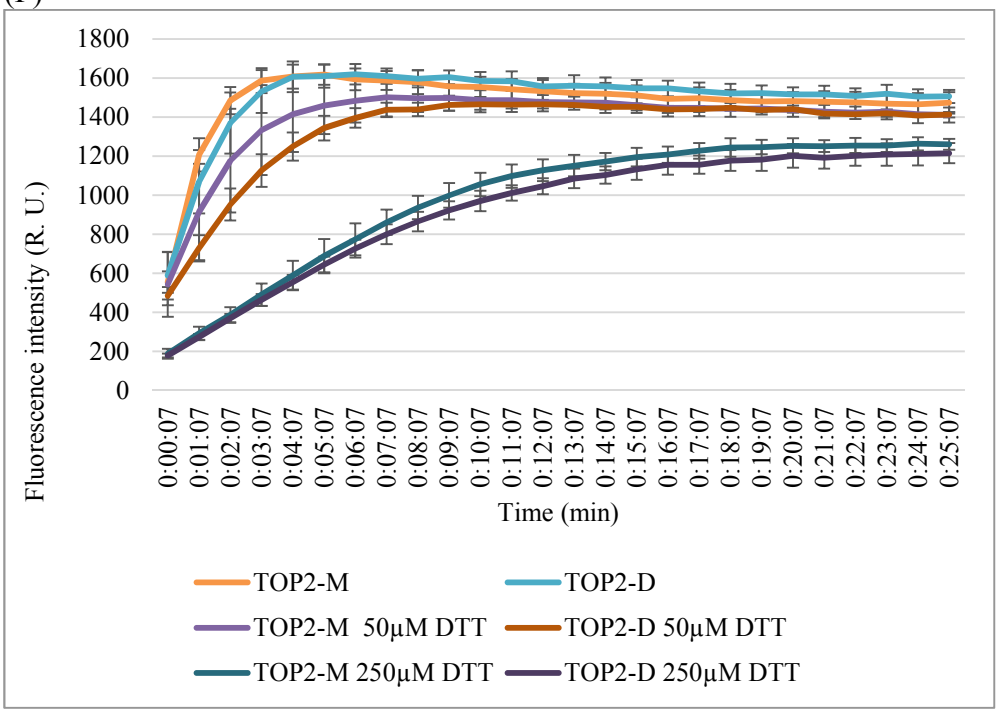
(D)


Relative enzyme activity (A. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
TOP2-M
TOP2-M 5mM DTT
TOP2-M 10mM DTT
Ctrl.

(E)


Relative enzymatic activity (A. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
**
**
TOP2-M
TOP2-D
TOP2-M 50uM DTT
TOP2-D 50uM DTT
TOP2-M 250uM DTT
TOP2-D 250uM DTT

(F)


Fluorescence intensity (R. U.)
1800
1600
1400
1200
1000
800
600
400
200
0
0:00:07
0:01:07
0:02:07
0:03:07
0:04:07
0:05:07
0:06:07
0:07:07
0:08:07
0:09:07
0:10:07
0:11:07
0:12:07
0:13:07
0:14:07
0:15:07
0:16:07
0:17:07
0:18:07
0:19:07
0:20:07
0:21:07
0:22:07
0:23:07
0:24:07
0:25:07
Time (min)
TOP2-M
TOP2-D
TOP2-M 50µM DTT
TOP2-D 50µM DTT
TOP2-M 250µM DTT
TOP2-D 250µM DTT

TOP1 and TOP2 are thiol-sensitive peptidases

Purified recombinant TOP1 and TOP2 were incubated in reaction buffer containing 20 μM of MCA-peptide fluorescent substrate. Upon cleavage, the quencher group of the fluorescent peptide is separated from the fluorophore. Cleaved MCA-peptide substrate emits detectable fluorescence. The activity was assessed at λ excitation of 328 nm and emission of 393 nm over the course of 25 min. Quantification of fluorescence intensity was expressed in the levels of fluorescence emission per minute and is shown as a relative enzymatic activity. Box plot is comprised of an upper quartile (gray) and a lower quartile (black). The line between the upper and lower quartile of box plot is the median. Asterisks represent statistical significance in enzymatic activity between the treated and untreated enzymes from two trials with 6 replicates (Student's T-test) (*$p < 0.05$ and **$p < 0.01$). The enzymatic activity of TOP1 under 5 and 10 mM DTT. Control constitutes reaction buffer containing no TOP enzyme.

(A) The enzymatic activity of TOP1 under 5 and 10 mM DTT. Control constitutes reaction buffer containing no TOP enzyme

(B) The relative enzymatic activity of TOP1 monomer and dimer after incubation in reaction buffer or reaction buffer containing 50/250 μM DTT.

(C) Fluorescence intensity of TOP1 monomer and dimer activity over time in reaction buffer containing no DTT or 50/250 μM DTT.

(D) The enzymatic activity of TOP2 under 5 and 10 mM DTT. Control constitutes reaction buffer containing no TOP enzyme.

(E) The quantification of TOP2 monomer and dimer activity regarding the relative enzymatic activity in the presence or absence of 50/250 μM DTT.

(F) Fluorescence intensity of TOP2 monomer and dimer activity over time in reaction buffer containing no DTT or 50/250 μM DTT.

ATP-based regulation of TOP activity does not occur in TOP1 and TOP2

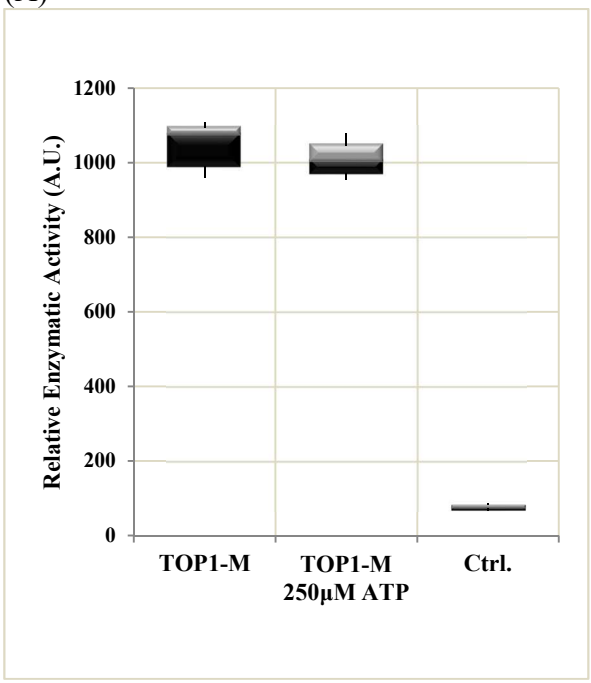
In mammalian system, evidence suggests that free ATP can regulate the activity of TOPs and potentially modulate peptide degradation (Portaro et al., 2001). Mammalian TOP interacts with ATP which inhibits peptidase activity and induces autophosphorylation (Portaro et al., 2001). The interaction of ATP on mammalian TOP is postulated to involve the HEXXH active site (Portaro et al., 2001). The HEXXH motif is characteristic of all TOPs and mediates zinc binding (David et al., 1998; Pierotti et al., 1990). TOP1 is localized to the mitochondria and may be subjected ATP-based regulation. ATP regulation of organellar TOP would introduce an added layer of enzymatic regulation. To determine whether free ATP modulates TOP1 activity, we examined the activity of the recombinant enzyme in the presence of 250 μM ATP. TOP1 activity levels are not affected by ATP (Figure 2-4 A).

TOP1 and TOP2 Maintain their Enzymatic Activity in a Wide Range of pHs

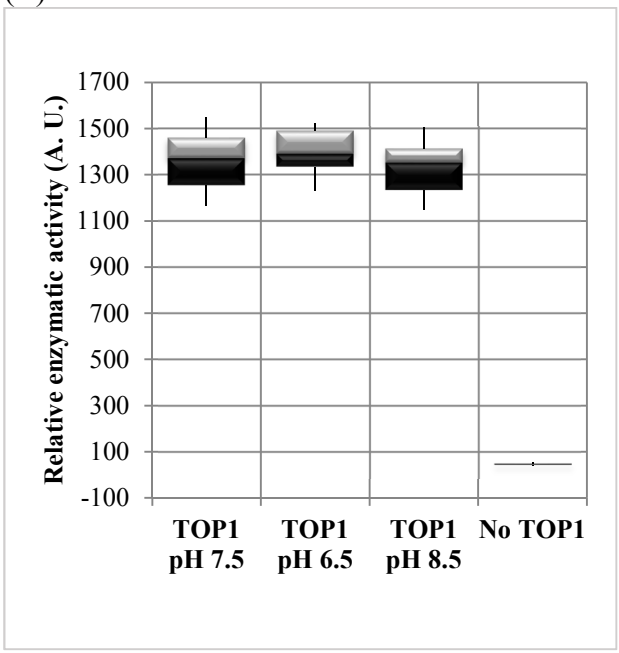
TOP1 possesses a signal peptide that facilitates the protein's localization to the chloroplasts and mitochondria. The function and activity of organellar enzymes are strongly influenced by pH changes in their environment, caused by fluctuations in the light quality and quantity (Buchanan, 1980; Scheibe, 1991). The stroma and mitochondrial matrix typically represent an alkaline environment. The pH of the stroma fluctuates from 6.2 to 4.6 in light versus darkness (Smith and Raven, 1979). In contrast, the pH of the cytosol is more stable and centers around 7.1 (Gout et al., 1992). To determine whether pH changes represent a potential regulatory mechanism of TOP activity, we examined the activity of recombinant TOP1-His and TOP2-His under a range of pH conditions. We found that the activities of TOPs on the fluorogenic substrate are impervious to changes of pH toward more acidic or basic values. TOP1 and TOP2 activities at pH 7.5 were unchanged from their activities at pH 6.5 or 8.5 (Figures 2-4 B, C).

Figure 2-4

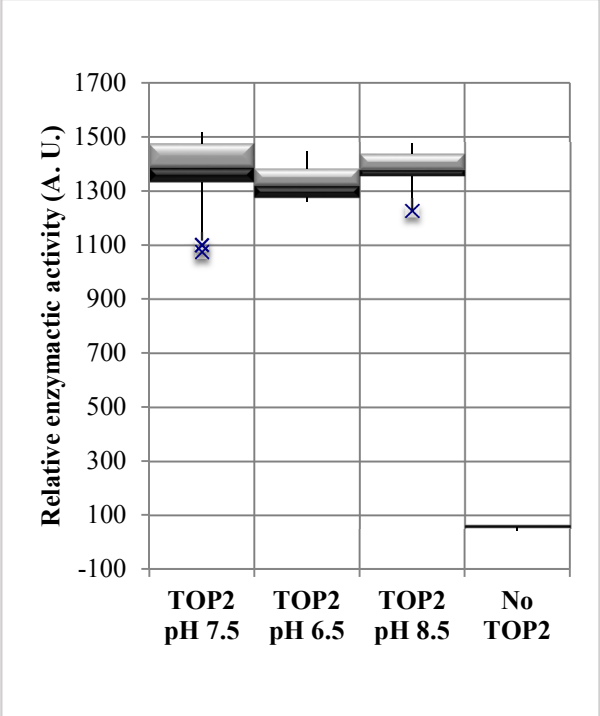
(A)


Relative Enzymatic Activity (A.U.)
1200
1000
800
600
400
200
0
TOP1-M
TOP1-M
250µM ATP
Ctrl.

(B)


Relative enzymatic activity (A. U.)
1700
1500
1300
1100
900
700
500
300
100
-100
TOP1
pH 7.5
TOP1
pH 6.5
TOP1
pH 8.5
No TOP1

(C)



Investigating additional modes of regulation of TOP1 and TOP2 activities.

Quantification of fluorescence intensity was expressed in the levels of fluorescence emission per minute and shown as relative enzymatic activity.

(A) The enzymatic activity of TOP1 in the presence or absence of 250 μM ATP. Control constitutes reaction buffer containing no TOP enzyme. The error bars are the standard deviation of two trials of 6 replicates.

(B) TOP1 activity in pH values of 6.5, 7.5, or 8.5. Reaction buffer with no enzyme is the negative control. The error bars are the standard deviation of 12 replicates.

(C) TOP2 activity in pH values of 6.5, 7.5, or 8.5. Reaction buffer with no enzyme is the negative control. The error bars are the standard deviation of 12 replicates.

TOP2 shares a substrate stabilization site with TOP1

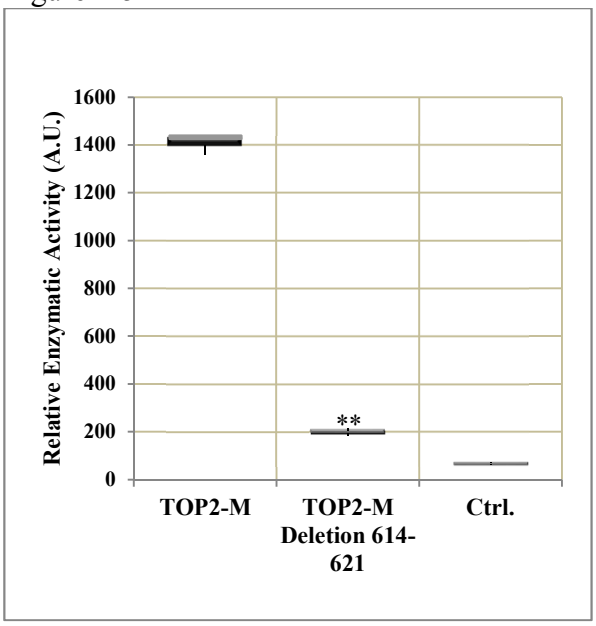
We explored how plant TOPs interact with their substrates. TOP1 and TOP2 have the same predicted substrate binding sites (Chapter 3). In TOP1, the H703 and Y709 amino acids of the SHIFAGGY site are involved in hydrogen bonding to TOP1 substrates (Kmiec et al., 2013). However, the functional significance of the site in TOP2 has yet to be elucidated. Due to sequence conservation and identity with TOP1, we hypothesize that the site is pivotal to TOP2 activity. The predicted binding site of TOP2 was deleted, and the activity was assessed. TOP2 amino acid residues 614-621 deletion mutants show a drastic reduction in enzymatic activity with an 87.49% decrease in activity levels (Figure 2-5).

The role of TOPs in oxidative stress responses

Prior evidence suggests that *TOP1* and *TOP2* peptidases contribute to plant defense against oxidative stress triggered by pathogens or abiotic factors (Polge et al., 2009; Moreau et al., 2013). TOP1 and TOP2 are both postulated to be involved in the degradation of peptides generated from the breakdown of oxidized proteins. TOP1 is thought to function in the context of the organelles while TOP2 is thought to operate in the cytosol. We aim to elucidate the potential involvements of TOP1 and TOP2 in plant oxidative stress responses.

To test whether TOPs are involved in oxidative stress responses, we examined the effect of oxidative stress inducers on *top* mutant lines. MV is a potent oxidative stress inducer. MV impairs photosynthesis by interfering with electron transport of the photosystems and by generating toxic superoxide anions (Farrington et al., 1973; Härtel et al., 1992; Krieger-Liszkay et al., 2011). The generation of ROS further damages the photosystems which inhibits growth, disrupts chloroplast homeostasis, and leads to PCD (Farrington et al., 1973). Also, MV induces lipid peroxidation and interferes with electron transport in mitochondria (Dodge, 1971; Palmeira et al., 1995). Furthermore, MV induces ROS production during seed dormancy and influences seed germination. To determine the influence of MV on the germination of *top* mutant lines, seeds were sown on medium containing MV, stratified for 2 days at 4°C in darkness and grown in

Figure 2-5



The putative substrate binding site of TOP2.

Purified recombinant TOP2 and TOP2 deletion mutant were incubated in reaction buffer containing 20 μM MCA-peptide substrate. Upon cleavage, the quencher group of the fluorogenic MCA-peptide is separated from the fluorophore emitting detectable fluorescence. The activity was assessed at λ excitation of 328 nm and λ emission of 393 nm over the course of 25 min. Quantification of fluorescence intensity was expressed in the levels of fluorescence emission per minute and shown as relative enzymatic activity. Data from two trials with three replicates. Asterisks represent statistical significance in enzymatic activity between the TOP2 and TOP2 deletion mutant (Student's T-test) (**$p < 0.01$).

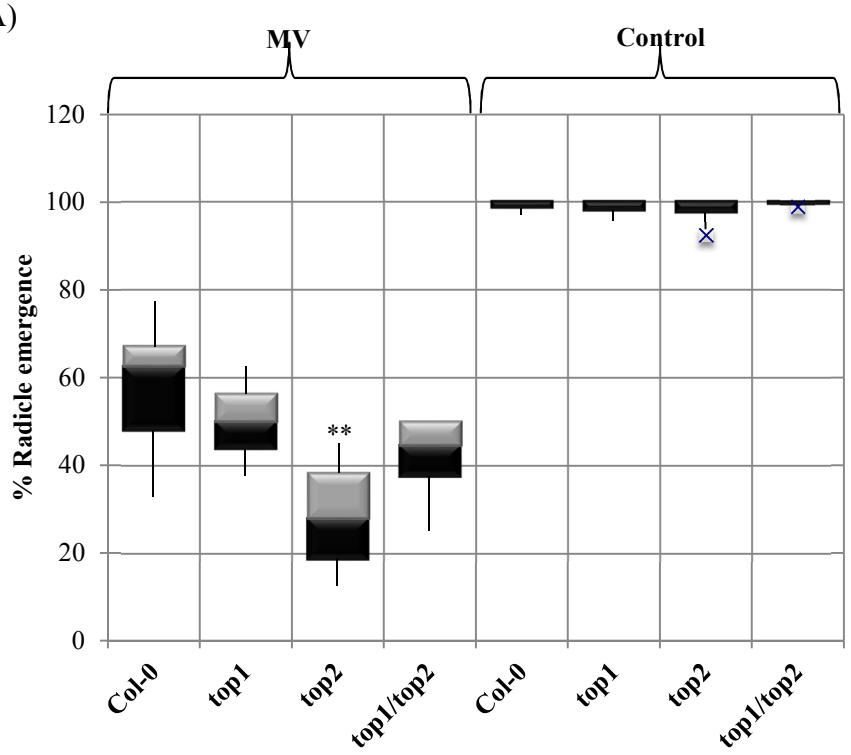
long-day (16 h) light conditions. Percent germination was assessed by monitoring the emergence of the radicle after 2 days of growth on 0.9 μM MV-containing medium for mutants and Col-0. In the presence of MV, the emergence of radicles in the *top2* mutant was significantly impeded (Figure 2-6 A). The germination rates of *top* mutants on control medium (no-MV condition) showed no significant differences from that of Col-0 seedlings. The results indicate that *TOP2* may positively modulate tolerance to MV exposure. MV applications on mature rosettes produced no differences between *top* mutants and Col-0. The photosystem efficiency of *top1*, *top2*, and *top1top2* did not differ after exposure to MV (Figure 2-6 C).

MV induces ROS production during seed dormancy. Short term MV treatment on dormant seeds within 6 hours resulted in improved germination rates by breaking dormancy (Farrington et al., 1973). To determine whether the oxidative stress resulting from ROS production upon prolonged exposure (48 hours) to MV during seed dormancy may be the cause of the hypersusceptible phenotype of the *top* mutants, *top2* and Col-0 were no longer stratified in the presence of MV. Instead, Col-0 and *top2* were stratified in water for 2 days and then seeded on MV-containing plates. We found that under these conditions the germination rate of *top2* increased drastically so that the difference between *top2* and Col-0 germination was no longer significant (Figure 2-6 B). Thus, the MV-mediated inhibition of the germination rate of *top2* only occurs when *top2* is exposed to the MV during seed dormancy.

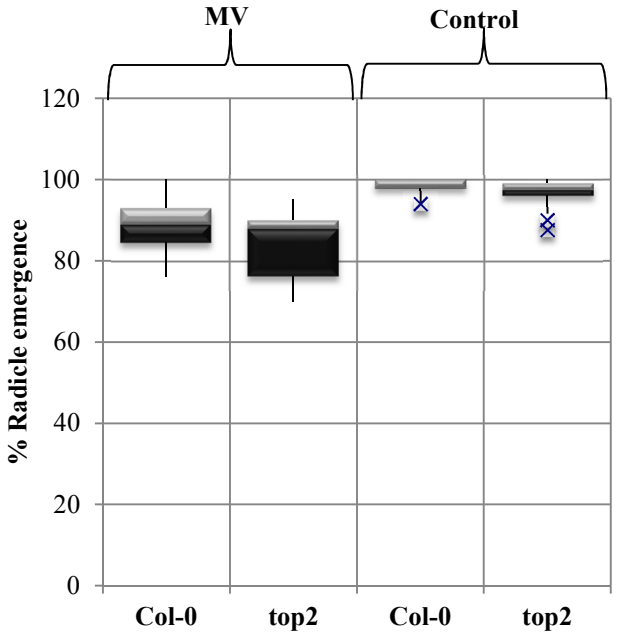
We examined the potential role of TOPs in mitigating the photo-oxidative stress induced by continual light and cold stress. Continual high light and cold stress induces photoinhibition and decreases photosystem efficiency. Col-0, *top1*, *top2*, and *top1top2* lines were transferred to constant light, and cold stress conditions. To investigate the function of TOPs in mediating responses to photooxidative stress, the Fv/Fm readings were examined to assess the integrity of photosynthesis. Col-0, *top1*, *top2*, and *top1top* lines all exhibited similar photosystem efficiency after exposure to oxidative stress (Figure 2-6 D). To further test the integrity of oxidative stress responses in *top* mutants, the effects of photo-oxidative stress induced high light was

Figure 2-6

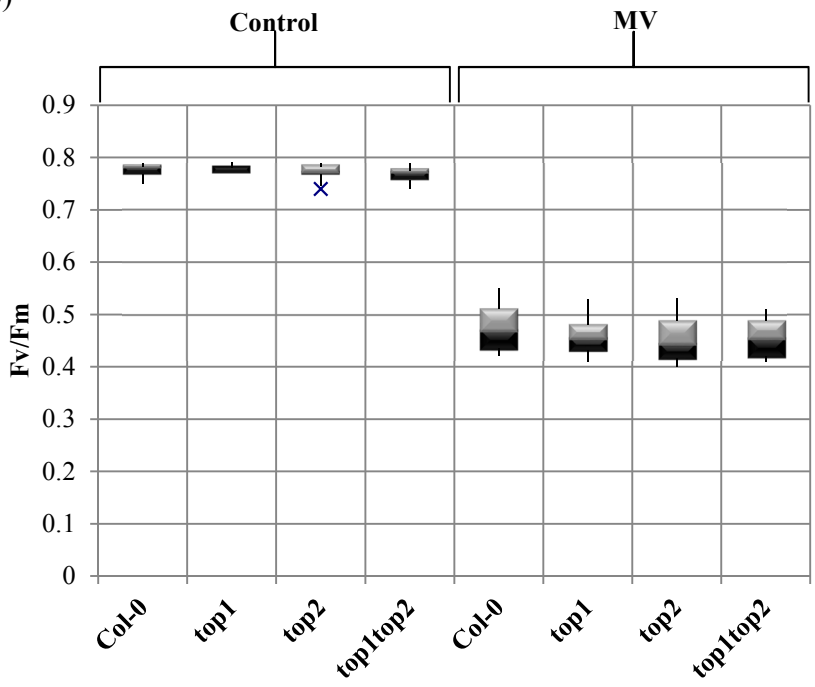
(A)


MV
Control
120
100
80
60
40
20
0
% Radicle emergence
**
Col-0
top1
top2
top1/top2
Col-0
top1
top2
top1/top2

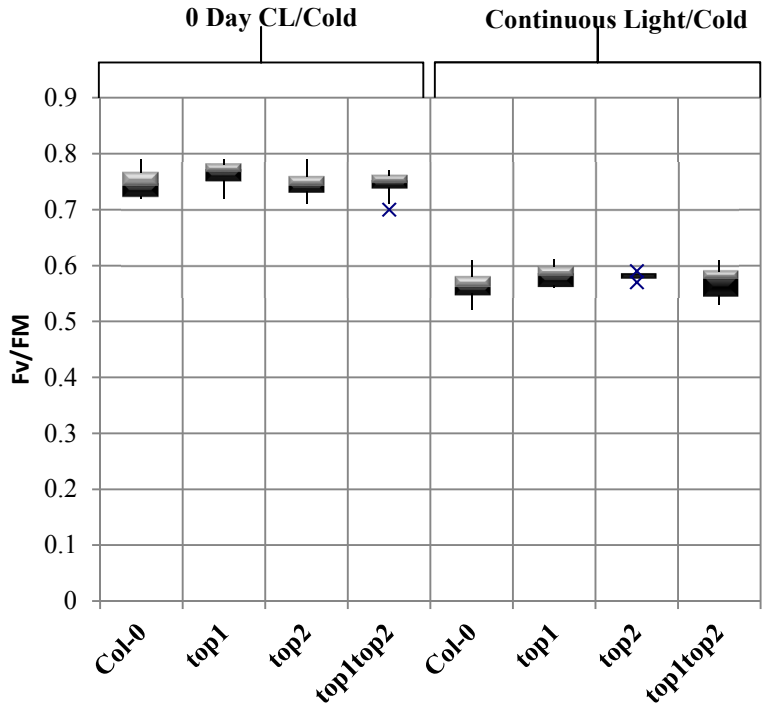
(B)


MV
Control
120
100
80
60
40
20
0
% Radicle emergence
Col-0
top2
Col-0
top2

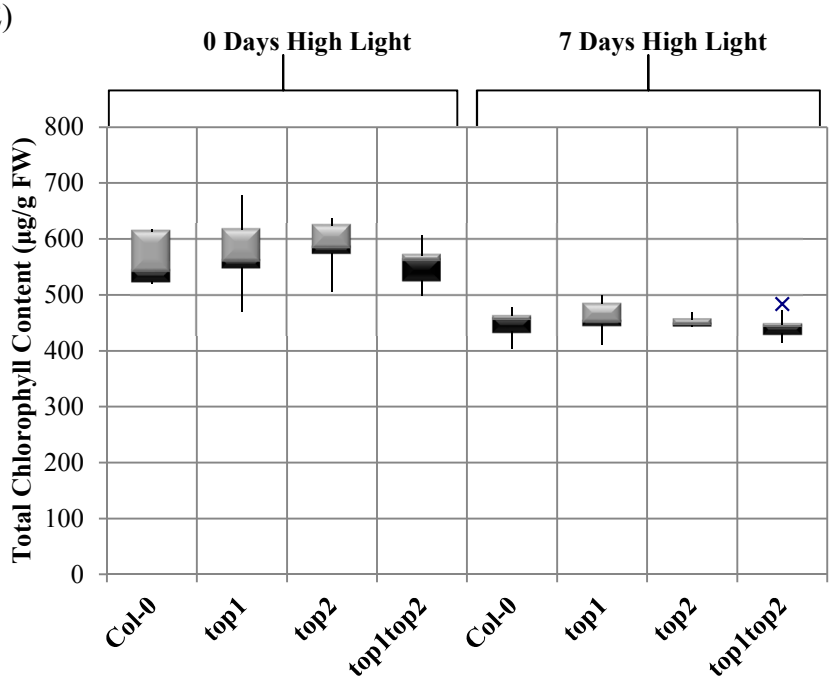
(C)


Control
MV
Fv/Fm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Col-0
top1
top2
top1top2
Col-0
top1
top2
top1top2

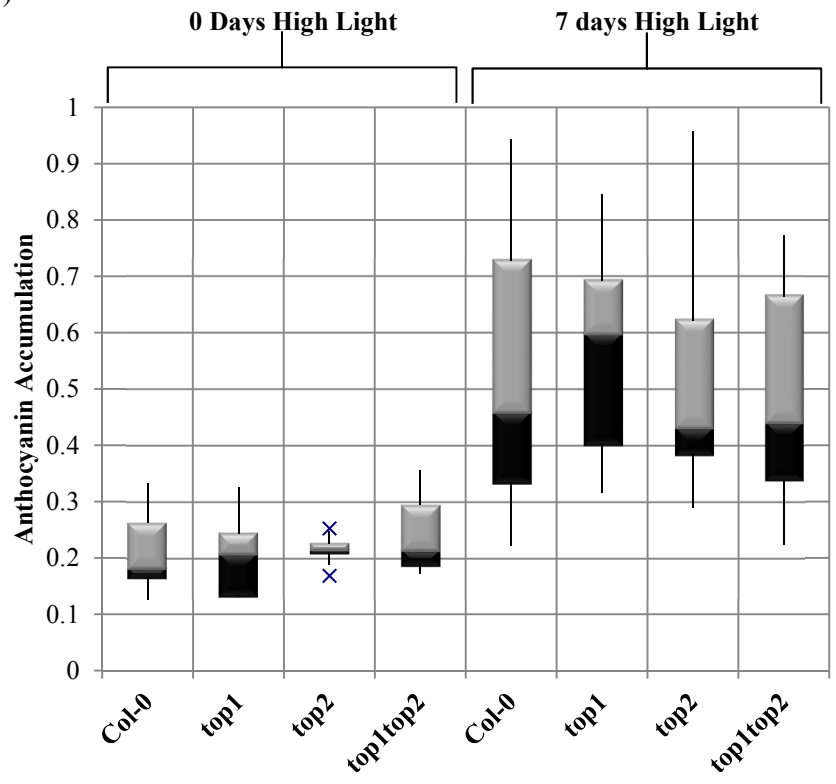
(D)


0 Day CL/Cold
Continuous Light/Cold
Fv/FM
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Col-0
top1
top2
top1top2
Col-0
top1
top2
top1top2

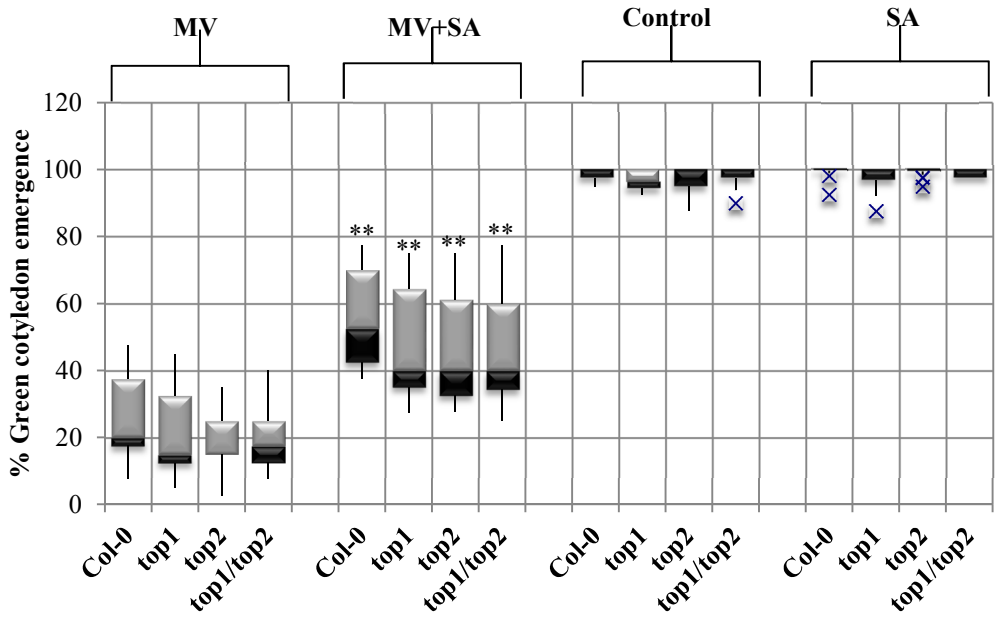
(E)


0 Days High Light
7 Days High Light
Total Chlorophyll Content (µg/g FW)
800
700
600
500
400
300
200
100
0
Col-0
top1
top2
top1top2
Col-0
top1
top2
top1top2

(F)


0 Days High Light
7 days High Light
Anthocyanin Accumulation
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Col-0
top1
top2
top1top2
Col-0
top1
top2
top1top2

(G)



Assessing the involvements of *TOP1* and *TOP2* in plant oxidative stress responses.

(A) Methyl viologen (MV) inhibits the radicle emergence of all lines. Seeds were stratified directly on MV-containing medium or no-MV (control) media. MV inhibition radicle emergence in the *top2* mutant is significantly different than MV inhibition of Col-0 radicle emergence. Seeds were stratified directly on MV-containing medium or no-MV (CTRL) media. Asterisks represent a statistical significance (T-test) of the difference between line performance in Col-0 and *top2* calculated from 6 replicates per treatment (*$p < 0.05$ and **$p < 0.01$).

(B) Stratification in water rescues the MV toxicity of *top2*. The seeds were stratified in water and then plated on MV (H_2O/MV) or no-MV plates (H_2O/ CTRL) plates. Data from 6 replicates per treatment.

(C) 3-4 week old leaves were syringe infiltrated with MV. Photosystem efficiency was assessed by assessing the quantum efficiency of photosystem II (Fv/Fm). Fv/Fm of leaves in untreated (water) or treated (MV).

(D) 2 week old plants were subjected to continuous light & cold stress. Photosystem efficiency was assessed by assessing the quantum efficiency of photosystem II (Fv/Fm). Fv/Fm of photosynthetic tissue was assessed before and after stress treatment.

(E) The chlorophyll content of 3-4 week old plants was assessed in high light conditions. Chlorophyll was extracted 0 days post-treatment and 7 days post-treatment.

(F) The anthocyanin content of 3-4 week old plants was assessed in high light conditions. Chlorophyll was extracted 0 days post-treatment and 7 days post-treatment.

(G) Exogenous SA alleviates the defects in green cotyledon emergence of both the top mutants and wild type Col-0. Asterisks represent a statistical significance (T-test) of the difference between line performance in MV+SA and in MV calculated from 6 replicates per treatment (*$p < 0.05$ and **$p < 0.01$).

assessed. Three-week old plant lines were transferred into high light conditions (800-900 μmol m^{-2} s^{-1}). After 7 days of high-light treatment, the chlorophyll content and anthocyanin accumulation were assessed. There was no difference in chlorophyll content and anthocyanin accumulation between Col-0 and *top* mutant lines (Figure 2-6 E, F). The results of the oxidative stress assays suggest that *TOPs* involvement in oxidative stress is likely related to the early development and may be limited MV-induced stress.

SA has a pronounced effect in limiting the oxidative damage induced by MV (Ananieva et al., 2002; Ananieva et al., 2004; Lee et al., 2010; Kim et al., 2003). Low levels of exogenous SA lessened the damage caused by oxidative stress through the modulation of antioxidant-related activities (Lee et al., 2010). Also, exogenous SA alleviated the effects of MV on photosynthesis (Ananieva et al., 2002). SA pretreatments resulted in reduced effects of MV such as lower hydrogen peroxide accumulation, less lipid peroxidation, and less cell death (Ananieva et al., 2002). To test whether SA-induced resistance to MV is compromised in *top* mutant lines, we sowed mutants and Col-0 seedlings on plates in the presence or absence of 10 μM SA and 0.95 μM MV and quantified their effects on the germination rate (Figure 2-6 G). The quantification of cotyledon emergence instead of radicle emergence was done to assess the recovery effect of SA on photosynthesis. We found that all lines exhibited normal germination rates in the presence of 10 μM SA, as determined by measuring the emergence of green cotyledons (Figure 2-6 G). We then examined whether SA beneficial effect on photosynthesis is functional in a *top* background by measuring the percentages of green cotyledons of seedlings grown in the presence of both 0.95 μM MV and 10 μM SA (Figure 2-6 G). We found that SA alleviated the adverse effect of MV on photosynthesis on all lines (Figure 2-6 G).

DISCUSSION

We elucidated the characteristics, enzymatic properties, and roles of *TOP1* and *TOP2* in the oxidative stress responses. We determined that TOPs are capable of forming dimers using three systems: the split luciferase system in protoplasts, which accounts for factors that may modulate TOPs interactions such as

the cellular redox environment and potential post-translational modifications, gel filtration chromatography, a method using purified proteins which allows for fine adjustments of the assay conditions, and Y2H, a method used assess the direct effects of SA on protein-protein interactions.

Our work shows that TOP2-TOP2 homodimers have the highest interaction affinity. TOP2-TOP2 homodimers show higher restored luciferase activity than TOP1-TOP1 homodimers and TOP1-TOP2 heterodimers. TOP1-TOP1 interactions have a seemingly weaker affinity in comparison to TOP2-TOP2 interactions. Despite the high sequence similarly between TOP1 and TOP2, TOP2 has an additional cysteine residue in the peptidase domain (Supplemental Figure 2-1). The additional cysteine residue may contribute to an additional disulfide bond, which in turn increases the strength of interaction. It may be that, analogous to thioredoxins in poplar (Chibani et al., 2012), the cysteine residue dictates TOP2's ability to form tight dimers. The additional cysteine residue of cytosolic TOP may be evolutionarily advantageous in a strong reducing environment. Alternatively, the weaker signal of TOP1-TOP1 interactions be attributed to the localization of TOP1. In protoplasts, the luciferase substrate readily accumulates in the cytosol. However, the amount of luciferase substrate translocated from the cytosol to the mitochondria and chloroplast is unclear. It is possible that TOP1-TOP1 interactions are as strong as TOP2-TOP2 interactions and a reduced amount of substrate in the organelles results in reduced luminescence and lower luciferase activity.

Our findings suggest that TOP1 and TOP2 are capable of forming heterodimers. The localization of TOP1-TOP2 interactions is not clear. TOP1 has a transit peptide that is absent in TOP2 (Chapter 3). Interestingly, recent proteomic studies have detected cytosolic TOP1 in 3 of the 3 independent assays (Ito et al., 2011). It may be that TOP1 may slightly accumulate in the cytosol where it can interact with TOP2. Alternatively, it is possible that TOP1 binds to TOP2 while in transit to the organelles. Mass spectrometry-based identification of chloroplastic proteins detected chloroplastic TOP2 (Kleffmann et al., 2004). Alternatively, it may be that the interactions may occur in the organelles. While the functional significance of

TOP1-TOP2 binding is unclear, we postulate that TOP1 and TOP2 may function cooperatively and may be co-regulated. Further studies are needed to determine the site of TOP1-TOP2 interactions and the functional significance of the heterodimer.

Assessing protein-protein interactions in protoplasts allowed for the observation of SA-signaling and SA-redox changes. In protoplasts, TOP2-TOP2 interactions appear to be weakened or reduced by exogenous SA. However, the intensities of MKK5-HopF2 (positive control) interactions are also reduced by SA. TOP2-TOP2 interactions show a concentration-dependent effect while the MKK5-HopF2 interactions show a strong non-concentration dependent effect of SA on luminescence. However, the influence of diminishing effects on the protein-protein interactions such as protoplast lysis cannot be completely disregarded. Therefore, further studies are needed to elucidate the potential effects of SA on TOP dimerization. Interestingly, TOP1-TOP2 interactions show a comparable trend of sensitivity towards SA as TOP2-TOP2, as the interaction intensity is reduced in a similar fashion. TOP1-TOP1 interactions appear to be resistant to exogenous SA. The full effect of SA on TOP1-TOP1 interactions is not entirely clear. The amount of exogenous SA translocated to the organelles is unknown. The insensitivity of TOP1-TOP1 interactions to SA may be in part due to limited SA accumulation in the chloroplasts and mitochondria during the time frame of the experiment. It may be that a higher concentration of SA is required to reach a threshold to induce an effect within the organelles. Future studies are necessary to elucidate the direct effects that SA or SA-induced redox changes have on organellar TOP dimerization.

Interestingly, we found that SA does not directly influence the interactions of TOP2-TOP2 in Y2H. Based on these results, the inhibitory effect of SA on TOP2-TOP2 interactions in protoplasts is not likely a result of direct SA-binding. It is plausible that the concentration-dependent effect of SA on TOP2 dimerization is a result of redox changes induced by SA. We speculate redox-changes may potentially cause the formation of the dimeric form throughout the oxidizing burst or the monomeric form during the reductive burst.

Our findings support the hypothesis that TOP1 and TOP2 form redox-sensitive disulfide bonds that may be regulated by the cellular redox state. TOP1 and TOP2 interactions are susceptible to reduction by two redox-reagents. The reducing agents drive the reduction of the dimeric form and results in the presence of predominantly the monomeric form. Furthermore, the major cellular redox regulatory molecule glutathione induces the dimeric and oligomeric forms of TOP2. Thus, analogous to other eukaryotic systems (Demasi et al., 2008), the oligomer formations of TOP1 and TOP2 are redox-sensitive. Furthermore, the ability of glutathione to regulate TOP interactions suggests that TOPs are broadly regulated by the cellular redox state. Interestingly, SA increases the content of redox-regulator molecules such as glutathione (Li et al., 2013; Herrera-Vasquez et al., 2015). It is plausible that the SA-induced changes in TOP dimer formation are a result of SA-induced redox changes.

The cysteine residues involved in the thiol-disulfide exchange, oligomerization, and S-glutathionylation have yet to be elucidated. The glutathione induced thiol-disulfide exchange suggests that the cysteine residues that interact with glutathione are also the cysteine residues that dictate the protein-interactions of TOP2. Furthermore, the ability of TOP1 and TOP2 to interact suggests the protein-interaction site is conserved. TOP1 and TOP2 have three conserved cysteine residues in the peptidase domain (TOP1 - C548, C611, and C699 and TOP2- C460, C523, and C611) (Supplemental Figure 2-1). It may be that these conserved cysteines are the critical cysteine residues involved in oligomerization and S-glutathionylation. Future studies will be needed to confirm the involvement of the conserved residues in TOP dimerization and multimerization. In mammalian systems, S-glutathionylation results in the hyperactive form of TOPs (Demasi et al., 2008). Our findings support that plant TOPs are S-glutathionylated. It may be that during certain levels of oxidative stress, increased oxidized glutathione content induces a hyperactive TOP form in plants. Interestingly, oxidized glutathione may promote the formation of the dimeric and multimeric forms of TOP2. The S-glutathionylated isoform and the resulting oligomers of TOP2 may have a protective function. S-glutathionylation is thought to prevent the irreversible protein oxidation of cysteine residues (Dalle-Donne et al.,

2009; Dixon et al., 2005; Ziegler, 1985). It may be that glutathione and the induced oligomeric forms of TOP2 shield the enzyme from irreversible modifications. Therefore, the activity of TOPs may continue to some extent even during oxidative stress. The functional significance of the multimeric form is unclear. It is plausible that analogous to mammalian systems, the multimer may be the inactive form of TOP. Future studies are needed to elucidate the function of the higher oligomeric forms.

Interestingly, our findings suggest that the monomeric and dimeric forms of TOPs may be active forms of the enzyme. TOP2 homodimer and TOP2 monomer do not significantly differ in activity. The higher form of TOP1 shows potential signs of reduced activity. However, the dimeric form still reaches comparable levels of activity to that of the monomer. It may be that the dimer is unstable and readily shifts to the active monomeric state over time. Alternatively, the dimeric form may still be an active state of the enzyme. There are multiple levels of redox regulations that are dependent on the varying disulfide bond formation in mammalian TOPs (Sigman et al., 2003). While in general, the dimeric form of TOPs in other systems are inactive, there is evidence that specific forms of the dimer have comparable activity to the monomeric form (Sigman et al., 2003). Certain dimeric forms of mammalian TOP have drastically reduced activity and are inactive while other dimeric forms are active (Sigman et al., 2003). The activity of the mammalian TOP dimer is dependent on which cysteine residues contribute to the formation of the dimer (Sigman et al., 2003). It is possible that a similar form of regulation occurs with plant TOPs.

Our work supports the hypothesis that TOP1 and TOP2 are thiol-sensitive peptidases. Incubation of plant TOPs with DTT results in a marked decrease in activity. As with other eukaryotic systems, incubation with DTT at concentrations greater than 1mM results in a drastic inhibition of peptidase activity. The inhibition induced by DTT is attributed to the thiophilicity of the catalytic zinc ion or possibly the disruption of intramolecular redox-sensitive bonds (Barrett and Brown, 1990; Morales and Woessner, 1977; Shrimpton et al., 1997). In mammalian systems, low level of thiols activates TOP activity (Shrimpton et al., 1997). Interestingly, the characterization of plant TOP activity shows differences in DTT regulation not seen in

mammalian TOPs. Levels of DTT that activate TOP activity in mammalian systems repress the activity of plant TOPs. Thus, plant TOPs have characteristics that distinguish them from other eukaryotic systems. Plant TOP1 and TOP2 are not as cysteine-rich as their mammalian TOPs counterparts. The comparatively fewer number of cysteine residues in TOP1 and TOP2 may potentially result in fewer intramolecular and intermolecular redox-sensitive bonds making the enzymes more susceptible to reduction by DTT. It is possible that the reduction of the intramolecular bonds may be deleterious to the tertiary structure of the enzymes. Alternatively, it may be that the catalytic zinc ion is possibly more exposed and accessible in plant TOPs than mammalian TOPs. It is possible that DTT may more readily interact with the catalytic zinc ion of TOP1 and TOP2, which in turn results in the pronounced inhibition. Further studies are required to determine the mechanisms of DTT inhibition of TOP activity in plants.

ATP-regulation of TOP activity in mammalian systems is postulated to be centered on the HEXXH active site motif (Portaro et al., 2001). The HEXXH motif dictates the binding of the catalytic zinc ion. It is thought that ATP binds to the catalytic zinc ion and modulates the activity of eukaryotic TOPs (Portaro et al., 2001). Interestingly, the ATP-based regulation may not occur in TOP1 and TOP2 despite the presence of the HEXXH motif. It is possible that the zinc ion is not responsible for ATP-regulation in mammalian systems as previously thought. Mammalian TOP substrate affinity is modulated by ATP (Portaro et al., 2001). Interestingly, mutations in the HEXXH site does not influence TOP substrate affinity (Rioli et al., 2003). Therefore, the ATP regulation of eukaryotic TOP activity may involve a separate site other than the HEXXH motif and the catalytic zinc ion. Alternatively, the accessibility of the zinc ion to ATP may be limited in TOP1 and TOP2. Nonetheless, our findings suggest that the regulation of plant TOPs is distinct from other eukaryotic TOPs as thiol-sensitivity and ATP-regulation differ.

Utilizing the 3D structure of TOP2, we predicted the enzyme's substrate binding site (Chapter 3). Deletion of the predicted substrate binding site of TOP2 results in drastic inhibition of peptidase activity. The results suggest that TOP1 and

TOP2 have a conserved substrate binding site. However, the influence of the deletion on protein folding is unclear. To confirm these findings, future studies are needed to determine the key amino acids within the SHIFAGGY site of TOP2 that mediate substrate binding. Interestingly, our study provides evidence that both the substrate binding site (Chapter 3) and the protein-interaction sites may be conserved between TOP1 and TOP2. The conservation of these binding sites suggests that TOP1 and TOP2 have similar substrate specificity and may potentially share substrates. Furthermore, the ability of TOP1 and TOP2 to interact suggests that TOPs may have cooperative function.

Our findings suggest that functional *TOP2* is needed to reduce the damage caused by MV. MV is an inducer of chloroplastic and mitochondrial oxidative stress. MV perturbs the photosynthesis resulting in a drastic reduction of photosystem efficiency and severe chloroplastic-derived oxidative stress (Babbs et al., 1989). Additionally, MV causes mitochondrial damage (Dodge, 1971; Palmeira et al., 1995). ROS accumulation has been found to promote seed germination. Interestingly, short-term exposure of seeds to MV increases ROS levels that are thought to contribute to the breaking of seed dormancy in *Arabidopsis* and *Helianthus* (Leymarie et al., 2012; Marino et al., 2012; Oracz et al., 2007). Interestingly, the loss of cytosolic TOP results in increased sensitivity to MV during radicle emergence. These findings are in line with prior research that suggest the cytosol is a major site for detoxification systems associated with organellar-derived stress (Mullineaux et al., 2000; Yabuta et al., 2004). Supporting this notion, overexpression of cytosolic oxidative stress related enzymes such as cytosolic glutathione reductase or cytosolic ascorbate peroxidase, results in increased resistances to MV (Lee and Jo, 2004; Pitcher et al., 1994). The *top1top2* mutant line may show less sensitivity to MV than *top2*. We speculate that the *top1top2* line potentially have differences in ROS or ROS signaling that may modulate cell death or germination during MV exposure.

Interestingly, there is no change in resistance to photo-oxidative stress inducers in mature leaves of *top* mutants. These findings suggest that TOP2's involvement in oxidative stress may be related to early development and seed germination.

Alternatively, it may be that plant TOPs function in limiting the oxidative stress induced by high-light, MV, or continuous light/cold, but it is masked by the multitude of oxidative stress responses in mature leaves. There are a plethora of mechanisms that plants employ to limit photo-oxidative stress (Foyer and Shigeoka, 2011). The roles of TOPs in oxidative stress may need to be explored in antioxidant deficient lines to elucidate any subtle functions of TOPs.

SA alleviated the deleterious effects of MV on germination in *top* mutants and Col-0 lines. SA amerloriates the toxicity of oxdative stress. SA modulates antioxidant systems, ROS-related enzymes, cellular ROS production, and nitric oxide accumulation in monocots and dicots (Gémes et al., 2011; Lee et al., 2010; Wang and Liu, 2012; Zhang et al., 2011). Furthermore, SA inhibits the damaging effects of MV by limiting ROS production and lipid peroxidation (Ananieva et al., 2002; Ananieva et al., 2004). It is possible that a similar mechanism is involved in the SA-mediated effect on MV toxicity in the seed lines. While the mechanisms behind the involvement of TOPs in MV resistance is unknown, it may be that TOPs have protective function by preventing the harmful accumulation of peptides.

In conclusion, our study has shown that TOP1 and TOP2 form homodimers and heterodimers. Our work suggests that TOP oligomerization is governed by redox-sensitive bonds. The intermolecular bonds of TOP1 and TOP2 are capable of being modulated by redox reagents. A primary redox regulator, glutathione, induces oxidative oligomerization of TOP2. Additionally, our findings have supported the identification of the substrate-binding site of TOP2. Similar to mammalian TOP, plant TOP1 and TOP2 are thiol-sensitive. However, TOP1 and TOP2 have distinct characteristics not seen in mammalian TOP as TOP1, and TOP2 activities are repressed by thiol-based reductants at concentrations lower than 1mM. Our work has also implicated *TOP2*'s role in oxidative stress.

Supplemental Figure 2-1

Protein: TOP1 Peptidase Domain
VMQHAKNRALREEVYRAYLSRASSGDLDNTAIIDQILK
LRLEKAKLLGYNNYAEVSMAMKMATVEKAAELLEKLRSASWDAAVQDME
DLKSFAKNQGAAESDSMTHWDTTFWSERLRESKYDINEEELRPYFSLPKVMD
GLFSLAKTLFGIDIEPADGLAPVWNNDVRFYRVKDSSGNPIAYFYFDPYSRPSE
KRGGAWMDEVVSRSRVMAQKGSSVRLPVAHMVCNQTPPVGDKPSLMTFRE
VETVFHEFGHALQHMLTKQDEGLVAGIRNIEWDAVELPSQFMENWCYHRDT
LMSIAKHYETGETLPEEVYKKLLAARTFRAGSFSLRQLKFASVDLELHTKYVP
GGPESIYDVDQRVSVKTQVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVL
SADAFSAFEDAGLDDIKAVKETGQRFRNTILALGGGKAPLKVFVEFRGREPSP
EPLLRHNG

Protein: TOP2 Peptidase Domain
VMQHAKNRALREEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGYRNY
AEVSMATKMATVEKADELLEKLRSASWDPAVQDIEDLKSFAKNQGAAEADS
LTHWDITFWSERLRESKYDINEEELRPYFSLPKVMDALFGLAKTLFGIDVVPA
DGVAPVWNSDVRFYCVKDSSGNPTAYFYFDPYSRPSEKRDGAWMDEVFSRS
RVMAQKGSSVRLPVAQMVCNQTPPVGDKPSLMTFREVETVFHEFGHALQHM
LTKEDEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAKHYQTGETLPE
NVYKKLLAARTFRAGSLSLRQLKFATVDLELHTKYMPGGAETIYEVDQRVSI
KTQVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDD
IKAVKETGQRFRNTILALGGGKAPLKVFVEFRGREPSPEPLLRHNG

The cysteine residues of the peptidase domains of TOP1 and TOP2.

The peptidase domain of TOP1 and TOP2 have three conserved cysteine residues. Yellow highlighted cysteines are the conserved Cysteine Residues: TOP1-C460, C523, C611 / TOP2-C548, C611, C699. TOP2 has an addition cysteine residue in the peptidase domain (underlined).

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CHAPTER 3

CHARACTERIZING THE ROLES OF TOP1 AND TOP2 IN PLANT IMMUNITY

ABSTRACT

Here, we describe our sequence comparison and functional characterization of plant thimet oligopeptidases (TOP). We show that TOP1 and TOP2 have high sequence identity with the highest sequence conservation in the peptidase domain. The other distantly related TOP-like proteins, TOPL1 and TOPL2, are dissimilar to TOP1. Nonetheless, as with the relationship between TOP1 and TOP2, the TOPL proteins have the highest sequence relatedness in the peptidase domain. Our analysis shows that TOP1, TOP2, TOPL1, and TOPL2, all contain an identical His-Glu-Phe-Gly-His active site motif. TOP1 and TOPL2 have a predicted transit peptide that is absent in TOP2 and TOPL1. Furthermore, we used the 3D structural data of TOP1 and TOP2 to predict the putative substrate binding site of the two peptidases. We explored the function of TOP1 and TOP2 in plant immunity. We provide evidence that TOP proteins are required for a fully functioning immune response to certain avirulent pathogens. The loss of both *TOP1* and *TOP2* results in increased susceptibility to specific avirulent *Pseudomonas syringae* strains. During the course of avirulent infection, cell death appears to be modulated in the *top* mutants. However, TOPs do not appear to modulate cell death induced by necrotrophic pathways. Additionally, we analyzed the expression profile of newly identified putative SABPs. We found five SABPs that are potentially co-regulated and up-regulated during plant immunity and biotic-related processes.

INTRODUCTION

Hormones are diverse signaling molecules with immense regulatory function in plants (Santner et al., 2009). In response to the perception of environmental stimuli, hormone signaling elicits the necessary changes for survival. Salicylic acid (SA) is a small molecule with tremendous importance in plant development and in the signaling involved in abiotic and biotic stresses (Vlot et al., 2009). SA is a phenolic hormone that plays a critical role in the regulation of biochemical processes throughout the plant life cycle (Vlot et al., 2009). The hormone induces cellular redox changes and transcriptional reprogramming, which mediate changes in protein activity, protein-protein interactions, and defense gene expression (Pieterse et al., 2012; Vlot et al., 2009). SA signaling is instrumental in activating a successful defense response and is a crucial component to plant disease resistance (Pieterse et al., 2012; Vlot et al., 2009). Successful induction of local immunity and systemic immunity requires a functioning SA-mediated signaling network. The successful recognition of conserved microbe associated molecular patterns (MAMP) in the case of MAMP-triggered immunity or effectors in the case of effector triggered immunity (ETI) resulting in immune activation is largely dependent on SA signaling (Delaney et al., 1994; Jones and Dangl, 2006; Pieterse et al., 2012; Tsuda et al., 2008). In addition, SA is involved in two major hallmarks of plant immune activation: the oxidative burst and programmed cell death (PCD) (Brodersen et al., 2005; Herrera-Vasquez et al., 2015). PCD, in the form of the hypersensitive response, limits the spread of infection (Morel et al., 1997). Impairment in SA signaling dampens immune responses and results in a weakened oxidative burst and PCD (Brodersen et al., 2005; Herrera-Vasquez et al., 2015).

Regulation of SA-signaling centers on the non-expressor of *PR* (NPR) family (Vlot et al., 2009). The monomerization and translocation of NPR1 into the nucleus are vital to the integrity of SA-dependent immune signaling (Vlot et al., 2009). NPR1 was found to specifically interact with SA, and may function as an SA receptor (Wu et al., 2012). The binding of SA to NPR1 is thought to directly facilitate the switch to the active form of the protein (Wu et al., 2012). SA binding is postulated to release the transactivation domain of NPR1 from inhibition (Wu et al., 2012). In addition,

evidence suggests that SA binding may facilitate the deoligomerization of NPR1 (Wu et al., 2012). Recently, NPR1 homologs, NPR3 and NPR4, were identified as SA receptors (Fu et al., 2012). SA binding modulates the interactions of NPR3 and NPR4 with NPR1 (Fu et al., 2012). Major forms of SA regulation occur through NPR3/NPR4 mediated NPR1 degradation (Fu et al., 2012). There also exists an NPR1 independent SA signaling pathway (Vlot et al., 2009). Despite the recent advances in SA targets and SA-signaling, the major regulators in NPR1-independent SA pathways are unclear. There are still many aspect of SA regulation that are unknown.

Large-scale high throughput screens have been used to identify potential SA-binding proteins (SABPs). Immuno-selection, in tandem with mass spectrometry-based identification, has identified 35 putative SABPs in *Arabidopsis thaliana* (Manohar et al., 2015). Protein microarray (PMA) is another valuable technique utilized for large-scale screens of SABPs (Manohar et al., 2015; Moreau et al., 2013). Recombinant plant proteins are immobilized on PMA slide where then thousands of proteins can be probed for SA-binding capacities (Manohar et al., 2015; Moreau et al., 2013). PMA have been used to identify over 100 *A. thaliana* proteins with the ability to bind to SA (Manohar et al., 2015; Moreau et al., 2013). To date, high-throughput screens have identified over 150 putative SABPs (Manohar et al., 2015; Moreau et al., 2013). The function of the majority of the newly identified SABPs in the context of SA-regulated processes and plant immunity is unknown. While SA is postulated to exert its effect on an array of proteins (Klessig et al., 2016), not all the newly identified proteins are thought to function in SA-regulated processes. The multitude of putative SABPs present researchers with a daunting task of selecting and characterizing proteins in SA-mediated processes.

PMA screens for proteins with SA-binding capacities identified thimet oligopeptidase 1 (TOP1) as a SABP (Moreau et al., 2013). TOP1 and its homolog, TOP2 were, selected for further characterization. The ability of TOP1 and TOP2 to bind to SA was verified with SA exclusion chromatography, SA-competitive binding assays, and surface plasmon resonance (Moreau et al., 2013). While both TOP1 and TOP2 bind to SA, the binding affinity of TOP1 and TOP2 are dissimilar (Moreau et

al., 2013). TOP1 has a higher SA binding affinity than TOP2 as the SA binding affinity of TOP2 is low in comparison (Moreau et al., 2013). Interestingly, the activities of TOP1 and TOP2 appear to be sensitive towards SA (Moreau et al., 2013). SA inhibits the activity of recombinant TOP1 and TOP2. (Moreau et al., 2013). Furthermore, applications of exogenous SA in plant extracts results in diminished activity on synthetic TOP substrate (Moreau et al., 2013).

Eukaryotic TOPs are zinc-dependent metallopeptidases of the M3 peptidase family (Pierotti et al., 1990; Orlowski et al., 1983; Orlowski et al., 1989; Tisljar et al., 1990). The His-Glu-Xaa-Xaa-His (HEXXH) active site motif is a defining feature of the metallopeptidases (David et al., 1998; Pierotti et al., 1990). The active site motif facilitates zinc-binding and is vital to the activity of TOPs (David et al., 1998; Pierotti et al., 1990).

Despite the presence of TOPs in plant systems and their immense importance in mammalian systems, their functions in plants are largely unknown. Plant TOPs are thought to be involved in oxidative stress responses (Polge et al., 2009). It is postulated that TOPs may be involved in the degradation of peptides that result from the breakdown of oxidized or damaged proteins (Kmiec et al., 2013; Polge et al., 2009). It is thought that TOP2 targets and degrades peptides downstream of the proteasome after oxidative damage in plant systems (Polge et al., 2009). Recent research has explored the involvement of TOP1 in organelle peptide processing. The characterization of *in vitro* TOP activity suggests that the peptidase may have a diverse range of substrates (Kmiec et al., 2013). *In vitro* TOP1 biochemical characterization has implicated degradation of cleaved transit peptides in the organelles (Kmiec et al., 2013). Free transit peptides must be degraded, as the accumulation is harmful to organellar function (Hof and Kruijff, 1995; Hof et al., 1993; Roise et al., 1986). The accumulation of free transit peptides results in mitochondria and chloroplast membrane damage, as the peptides may be imbedded to the lipid bilayer or interact with proteins (Hof and Kruijff, 1995; Hof et al., 1993; Roise et al., 1986). In addition, TOP1 is thought to be involved in the complete degradation of oxidized/damaged protein and limit peptide accumulation (Kmiec et al., 2013). It is postulated that TOP1

may be involved in an array of proteolytic events due to the broad specificity of the enzyme (Kmiec et al., 2013). The function of mammalian TOPs in mammalian systems has been shown to be vital to a plethora of different functions such as apoptosis, immunity, oxidative stress tolerance, hormone signaling and processing, peptide processing, and maintenance of cellular homeostasis (Chu and Orlowski, 1985; Dahms and Mentlein, 1992; Glucksman et al., 1992; Kloetzel et al., 2004; Koike et al., 1999; Piva et al., 2000). The functions of plants TOPs is unclear.

In this study we describe our sequence comparison and functional characterization of plant TOPs. We explore the functions of TOP1 and TOP2 in plant immunity and cell death. Furthermore, we analyze the expression profile of genes encoding the SABPs identified with the protein-microarray. Our transcriptional analysis found five SABPs that are potentially co-regulated and up-regulated during plant biotic stress and immune elicitors.

MATERIALS AND METHODS

Predictive Substrate Binding Site

Protein Database Bank files of the three-dimensional structure of TOP1 and TOP2 were processes through neural network protein-protein interaction site predictor software (Chen and Zhou, 2005; Zhou and Shan, 2001). Consensus protein-protein interaction site predictor (con-PPISP) program was used to assess the protein interaction sites based on structure (Chen and Zhou, 2005; Zhou and Shan, 2001). No three-dimensional structural data are available for TOP-like 1 (TOPL1) and TOPL2, thus, the two proteins were excluded.

Sequence Comparison

Sequence alignment was performed by NCBI Sequence alignment tools (Altschul et al., 1990) (https://blast.ncbi.nlm.nih.gov/). The reference sequence was TOP1 (At5g65620). The sequences compared were TOP2 (AT5G10540), TOP-Like 1 (TOPL1) (AT1G67690), and TOPL2 (AT5G51540). The C-terminal peptidase domain of TOP1 was compared with the peptidase domain of TOP2, TOPL1, and TOPL2. The N-terminal sequences (excluding the transit peptide) of TOP2, TOPL1, and TOPL2, were compared to the amino acid sequence of TOP1. No significant sequence

similarity was found between the N-terminus of TOP1 and TOPL1/TOPL2. Sequence alignments were as seen in Supplemental Figure 3-1.

Predictive Transit Peptide of plant TOPs

Transit peptides of TOP1, TOP2, TOPL1, and TOPL2 were examined using subcellular location prediction program TargetP 1.1 (Emanuelsson et al., 2000; Nielsen et al., 1997).

Plant Material and Pathogen Infections

Seeds were surface-sterilized utilizing 25% bleach and .01% tween solution. After surface sterilization, the bleach solution was pipetted out and the seeds were rinsed with water five successive times. The seeds were sowed unto metro-mix soil. Afterwards, the seeds were incubated in complete darkness at 4°C for 48 hours. Following the dark incubation, the plants were moved to growth chambers. The growth chamber temperature was set at 23 ± 2 °C, with light intensity of 200 μmol $m^{-2}s^{-1}$. The plants were grown for 3 to 4 weeks and utilized for successive assays. The T-DNA insertion lines utilized for the experiments were t*op1-339*, *top1-439, top2-127*, *top2-727*, *top1-339top2-127*, *top1-339top2-727* as previously described (Moreau et al., 2013).

Pseudomonas syringae pv. *tomato* (*Pst*) strains were cultured on Kings's B (KB) medium at 28°C-29°C. Afterwards, *P. syringae* pv. *tomato* strains were grown in KB liquid culture and then plated onto KB agar plates. The pathogen was suspended in $MgCl_2$ buffer solution at concentrations ranging from 10^5-10^6. The pathogen was gently syringe infiltrated into the abaxial side of the leaf using a needless syringe. Four leaves were infiltrated per plant. Leaves were harvested for colony forming unit (CFU) during designated time points. The collected leaf tissue were completely macerated in $MgCl_2$ buffer using a mechanical paint-shaker. The 10 μl of the macerated lysate was plated on KB agar at dilutions ranging from 10^0 to 10^{-7}. The bacteria was allowed to grow at room temperature. Colonies were counted 48 hours after plating. For relative death quantification, leaves were harvested at designated time-points with a razorblade. Extracted leaves were immediately photographed afterwards. The cell death of the lines assessed utilizing the scale seen in Figure 3-2.

Pectobacterium carotovorum was grown on lysogeny broth (LB) media. The inoculations and bacterial tiller assays were done as previously described (Singh et al., 2012). The concentration used for infection was 2×10^3 CFU/mL. Inoculations with pathogens was performed in a 10 mM $MgSO_4$ buffer solution. 3-4 week old plants were utilized for the assay. After inoculation, leaves were collected 2 DPI for bacterial tiller assay. Dilutions of bacteria were plated on LB agar. CFU was calculated 2 days later. For the *P. carotovorum* ion leakage assays, 3 week old plants were inoculated with initial inoculum of 1×10^8 CFU/mL. After inoculation, 6 leaf discs were cut and rinsed. Afterwards, the discs were floated on 12mL of distilled deionized water. The ion leakage was assessed at 0 DPI and 2 DPI via ion conductance.

Hyphae of *Sclerotinia sclerotiorum* were grown on potato dextrose agar for 2 days. A small agar plug of the pathogen was transferred to fresh potato dextrose agar. The hyphae was allowed to grow for 2 additional days. Afterward, 10mM diameter agar plugs were placed on detached leaves of 4 week old *A. thaliana.* Two days after infection, the leaves were photographed. The diameter was assessed by ImageJ software, a public domain imaging software provided by the National Institute of Health (NIH) (Scneider et al., 2012).

Quantitative Real Time PCR

RNA extraction was performed utilizing the TRIZOL RNA isolation protocol according to manufacturer instructions (Thermo Fisher). cDNA was obtained via reverse transcription of the extracted RNA utilizing Avian Myeloblastosis Virus reverse transcriptase manufacturer instruction (Promega). cDNA was stored at -20 °C until further use. iTaq Universe SYBR Green reaction mixture was utilized for the Quantitative Real Time PCR (QRTPCR) in accordance to manufacturer instructions (Bio-Rad). Bio-Rad RTPCR thermocycler and optical detection module was utilized for QRTPCR experiments. Quantitative Real Time PCR (QRTPCR) analysis was done according to Schmittgen and Livak, 2008. The internal control for all QRTPCR reactions study was protein phosphatase 2A (*PP2A*).

For flg22 assays, seedlings of wild-type, *top2-127, top1-339, top1-439,* and *top1-3top2-1* were grown on MS liquid media solution in long day (16 hour light and

8 hour darkness) conditions. The MS solution was extracted and replaced with MS solution with 1uM flg22 or control (water). Seedlings were frozen and grown after 24 hour incubation. The expression of *TOP1*, *TOP2,* and *PR1* was examined. Assessment of *PR1* expression was utilized as a SA marker gene. RNA and cDNA were obtained as described above.

Genevestigator transcriptional analysis

Genevestigator is a meta-analysis software of transcriptomes (Hruz et al. 2008). The analysis toolbox was used to analyze the expression of the genes encoding the SABPs found in the protein microarray screen (Moreau et al., 2013). Probes were available for all but one gene (AT5G08680). Therefore, AT5G08680 was excluded from the study. The parameters for the transcriptional analysis pertain to SA-regulated processes and plant immunity. In addition, the expression of the SABPs were analyzed for coregulation and upregulation during immune related processes utilizing Genevestigator's BICLUSTER program. The SABPs that show upregulation and coregulation during plant immune-related processes were selected for further analysis.

Predicted localization and metal binding sites of the five SABPs.

The sequences of the five SABPs identified from the Genevestigator transcriptional analysis were analyzed. The predicted localization was examined via TargetP. Peroxisomal targeting sequences (PTS) were assessed through the identification of the serine-lysine-leucine motif. In addition, the potential conserved sites that may dictate SA binding sites across the five SABPs were analyzed via protein BLAST. Metal binding site was predicted using SeqCHED Prediction servers (Levy et al., 2009).

RESULTS

Sequence analysis of plant TOPs

There are four TOP-like proteins of the M3 family in *A. thaliana.* The TOP-like proteins in *Arabidopsis thaliana* have the definitive HEXXH motif characteristic of metallopeptidases (Table 3-1). The active site motif is conserved in all four proteins and contains an identical His-Glu-Phe-Gly-His motif (Table 3-1). Utilizing consensus

Protein-Protein Interaction Site Predictor (con-PPISP) software, the 3D structure of TOP1 and TOP2, and sequence homology, we searched for candidate substrate bindings sites in TOP1 and TOP2. The analysis of the 3D structure of plant TOPs identified the SHIFAGGY sequence as the putative peptide binding sites of TOP1 and TOP2 (Table 3-1). Interestingly, the predicted substrate binding site of TOP1 is identical to the predictive substrate binding site of TOP2 (Table 3-1). TOPL1 and TOPL2 do not have the SHIFAGGY amino acid sequence and do not appear to have a related sequence. There is no 3D structural data for TOPL1 and TOPL2 therefore their predicted substrate binding site could not be identified.

We analyzed the sequences of the four TOPL peptidases using TOP1 as a frame of reference. The analysis was based on the sequence alignments seen in Supplemental Figure 3-1. TOP1 and TOP2 have high sequence identity in the amino acid level. The C-terminal peptidase domain of TOP1 and TOP2 has the highest sequence identify at 94% (Table 3-1). Outside the C-terminal domain, the aligned N-terminal sequence of TOP2 has 91% identity with TOP1 (Table 3-1; Supplemental Figure 3-1). TOP1 has an N-terminal transit peptide that is absent in TOP2 (Table 3-1). The sequence identity between the two TOPL proteins and TOP1 are greatest at the C-terminal peptidase domain. TOPL1's C-terminal peptidase domain has 28% sequence identity with TOP1 (Table 3-1). TOPL1 N-terminus is dissimilar to TOP1 and minimal sequence conservation. The sequence identity of between TOP1 and TOPL2 at the C-terminal peptidase domain is 27% (Table 3-1). As with TOP1, TOPL2 contains a predictive transit peptide sequence (Table 3-1).

Characterizing the roles of TOP1 and TOP2 in plant immunity

In order to determine whether *TOP*s are involved in plant immunity, *top* mutant lines were subjected to various pathogen infections. In this study, we examined the integrity of resistance towards hemi-biotrophic pathogens and necrotrophic pathogens. The model pathogen for the hemi-biotrophs was *P. syringae* pv. *tomato* (*Pst*). The strains of *Pst* utilized were *Pst* Δ*hrcQU*, *Pst avrRpt2, Pst avrRps4, Pst avrPphB*. The double mutants *top1-339top2-1/27* and *top1-339top2-727* showed

Table 3-1

Protein	HEXXH Active Site Motif	Peptidase Domain Sequence Identity	N-Terminal Sequence Identity	Transit Peptide (TargetP)	Predicted Substrate Binding Site (PPIP)
Reference TOP1 (AT5G65620)	HEFGH	--	--	Present	SHIFAGGY
TOP2 (AT5G10540)	HEFGH	94%	91%	Absent	SHIFAGGY
TOPL1 (AT1G67690)	HEFGH	28%	Minimal	Absent	N/A
TOPL2 (AT5G51540)	HEFGH	27%	Minimal	Present	N/A

The amino acid sequence comparison of the four thimet oligopeptidase (TOP)-like (TOPL) proteins in *Arabidopsis thaliana*

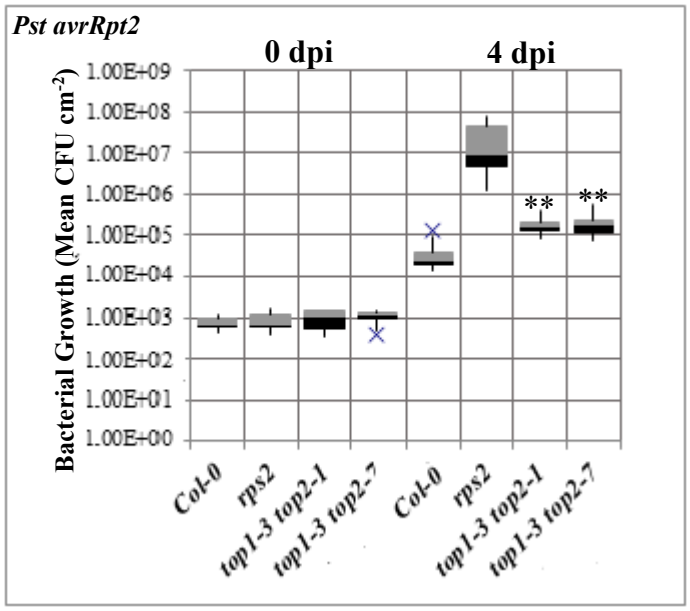
The four TOPL proteins in *Arabidopsis thaliana* have a conserved HEXXH active site characteristic of thimet oligopeptidases and members of the M3 family of peptidases. In addition, the active sites of the peptidases are all identical (HEFGH). The sequence identity of TOP2, TOPL1, and TOPL2 were processed using protein blast with TOP1 as a reference of comparison. The alignments are as seen in Supplemental Figure 3-2. TOP1 and TOP2 are highly similar with 94% sequence identity in the peptidase domain and 91% similarity in the N-terminal (without the transit peptide). TOP1 and TOPL1 had 28% sequence identity in the peptidase domain. TOP1 and TOPL1 N-terminal sequence comparison show minimal sequence similarity. TOP1 and TOPL2 have 27% sequence identity in the peptidase domain. TOPL2 N-terminal and TOP1 N-terminal sequences have minimal sequence identity. TOP1 and TOPL2 contain a transit peptide sequence that is absent in TOP2 and TOPL1. Utilizing the Consensus Protein-Protein Interaction Site Predictor network method, the substrate interaction site was predicted in TOP1 and TOP2. No structural data for TOPL1 and TOPL2 is available and therefore they were excluded. TOP1 and TOP2 have identical putative substrate binding sites.

enhanced susceptibility towards *Pst avrRpt2* at 4 days post-inoculation (DPI) (Figure 3-1 A). Infections with *Pst avrRps4* appear to show signs of compromised resistance in *top* double mutants at the later stages of infection. *top1-339top2-127* double mutants showed potentially enhanced growth of *Pst avrRps4* relative to Col-0 at 6 DPI (Figure 3-1 B). The resistance in *top* single mutants are not compromised towards *Pst avrRps4* (Figure 3-1 B). *TOP* knockout mutants do not have increased susceptibility towards *Pst avrPphB* and *Pst* Δ*hrcQU*, an avirulent type-III secretion system mutant (T3SS) strains of *Pst* (Figure 3-1 C, D). The signs of compromised resistance at later stages of infection with *Pst avrRpt2* and *Pst avrRpt4* may be related to the necrotrophic phase of *Pst*. To test this hypothesis, *top* lines and wild-type lines were infected with necrotrophic pathogen *Pectobacterium carotovorum.* After 2 DPI, the bacterial growth in *top* leaf tissue and wild-type Col-0 showed no statistical difference (Figure 3-1 E). Resistance to *P. carotovorum* was uncompromised in *top* knockout lines.

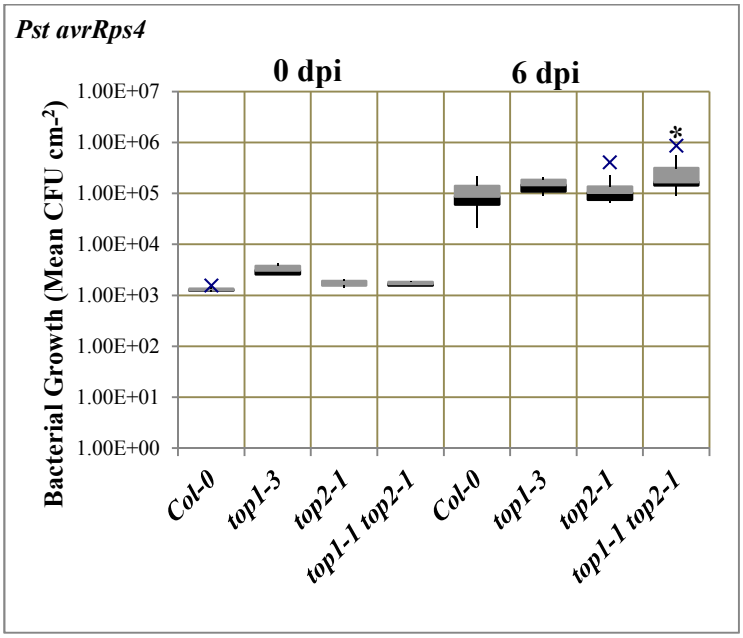
We examined the progression of cell death during infection with three different pathogens. *Pst avrRpt2* triggers reactive oxygen species (ROS) burst and PCD in *Arabidopsis thaliana. Pectobacterium spp.* and *Sclerotinia sclerotiorum* are broad-host range necrotrophic pathogens that induce robust cell death (Ma et al., 2007; Mbengue et al., 2016). If TOPs have a role in the regulation of oxidative stress, it is plausible that during the course of infection, the cellular death responses may be perturbed. The cell death induced by *Pst avrRpt2* shown signs of variation from one another in *top1*, *top2*, and *top1top2* double mutant lines (Figure 3-2 A). The loss of cytosolic *TOP2* appears to result in an observed increased amount of cell death relative to wild-type (Figure 3-2 A). In *top1-3* knockout mutant, cell death after infection appeared dampened in comparison to wild-type (Figure 3-2 A). In *top1-339top2-127* and *top1-339top2-727* lines, the double mutant displays an intermediate phenotype of the *top1* and *top2* mutant similar to that of Col-0 (Figure 3-2 A).

Figure 3-1

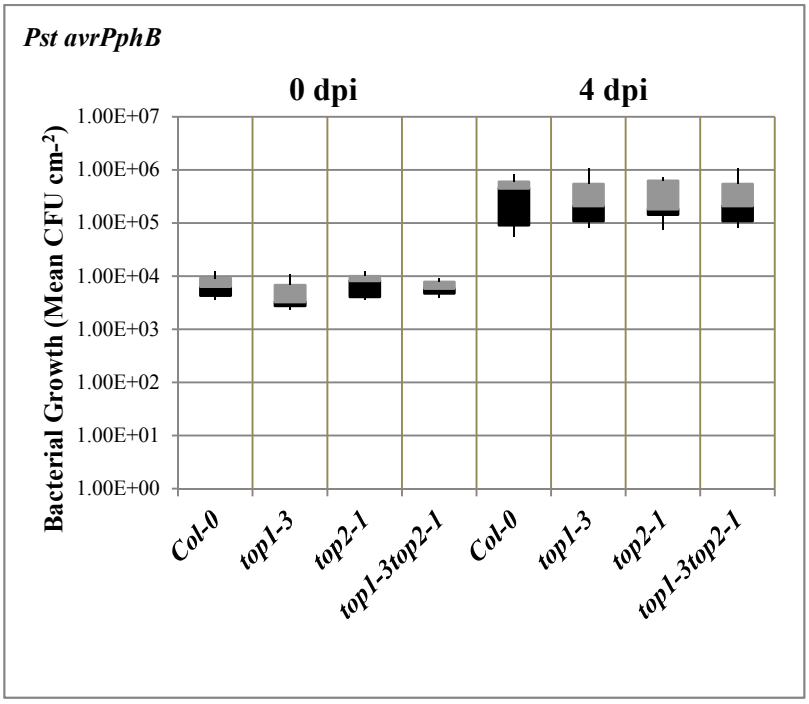
(A)


Pst avrRpt2
0 dpi
4 dpi
Bacterial Growth (Mean CFU cm-2)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
**
**
Col-0
rps2
top1-3 top2-1
top1-3 top2-7
Col-0
rps2
top1-3 top2-1
top1-3 top2-7

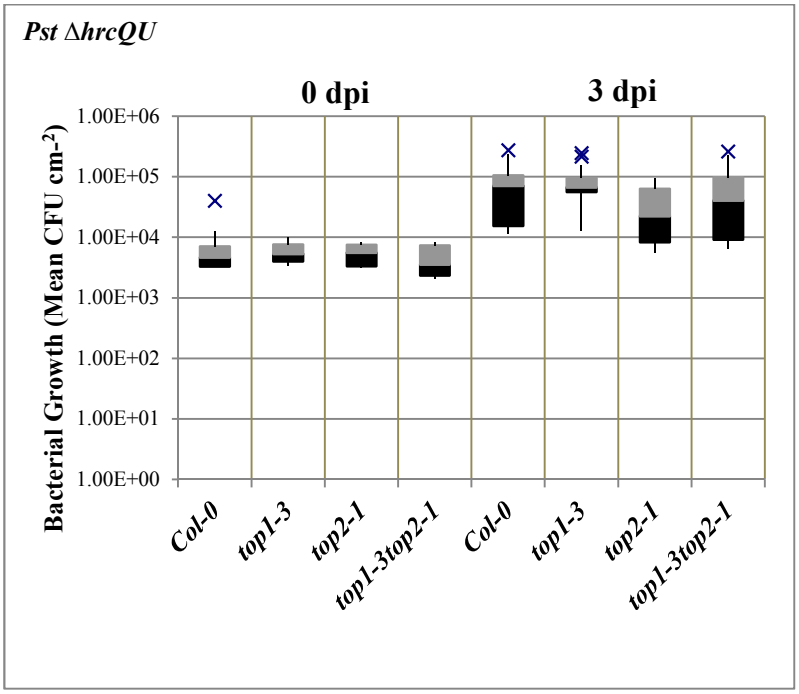
(B)


Pst avrRps4
0 dpi
6 dpi
Bacterial Growth (Mean CFU cm-2)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
*
Col-0
top1-3
top2-1
top1-1 top2-1
Col-0
top1-3
top2-1
top1-1 top2-1

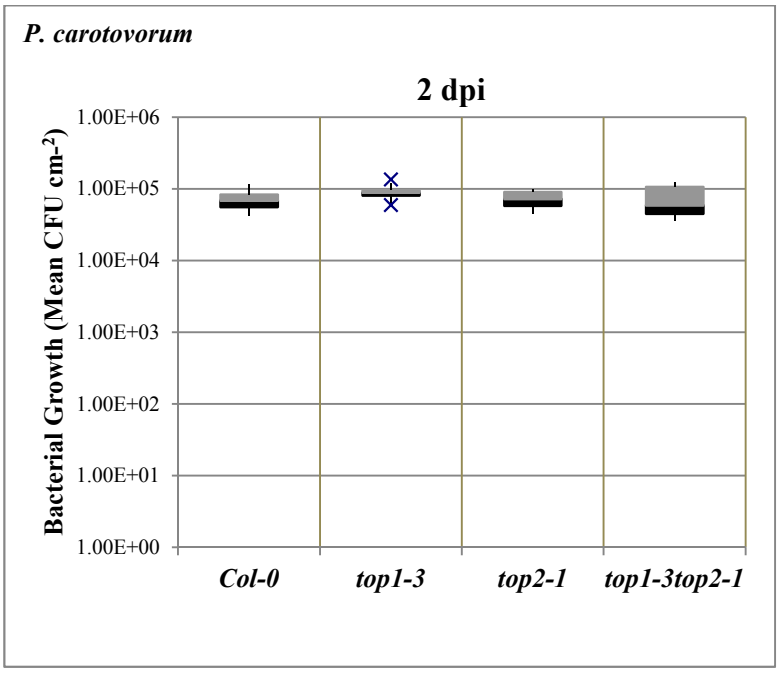
(C)


Pst avrPphB
0 dpi
4 dpi
Bacterial Growth (Mean CFU cm-2)
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Col-0
top1-3
top2-1
top1-3top2-1
Col-0
top1-3
top2-1
top1-3top2-1

(D)


Pst ΔhrcQU
0 dpi
3 dpi
Bacterial Growth (Mean CFU cm-2)
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Col-0
top1-3
top2-1
top1-3top2-1
Col-0
top1-3
top2-1
top1-3top2-1

(E)



Characterizing the involvement of TOP1 and TOP2 in plant immunity.

(A) Disease progression during infection with *Pst avrRpt2*. CFU counts were assessed immediately after inoculation and 4 days post-inoculation (DPI). The *top1top2* double mutant lines have increased susceptibility to *Pst avrRpt2*. Values derived from three trials, with five replicates per treatment. Asterisks represent statistical significance between Col-0 and top mutant lines (Student's T-test) (*$p < 0.05$ and **$p < 0.01$).

(B) Infections with *Pst avrRps4*. CFU counts were determined after initial inoculation and 6 days post-inoculation. The *top1top2* double mutant has higher susceptibility to *Pst avrRps4*. Values derived from two trials, with five replicates per treatment. Asterisks represent statistical significance between Col-0 and top mutant lines (Student's T-test) (*$p < 0.05$ and **$p < 0.01$).

(C) *Pst avrPphB* strain was inoculated into the plant lines. The bacterial titers were assessed after initial inoculation and 4 days post-inoculation. The CFU count does not differ between lines. Data from two trials, with four replicates per treatment.

(D) *Pst* ΔhrcQU strain was used to inoculate Col-0 and top mutant lines. Bacterial titer was determined after initial inoculation and 3 days post inoculation. Bacterial growth does not differ between the test lines. Data from two trails, with four replicates per treatment.

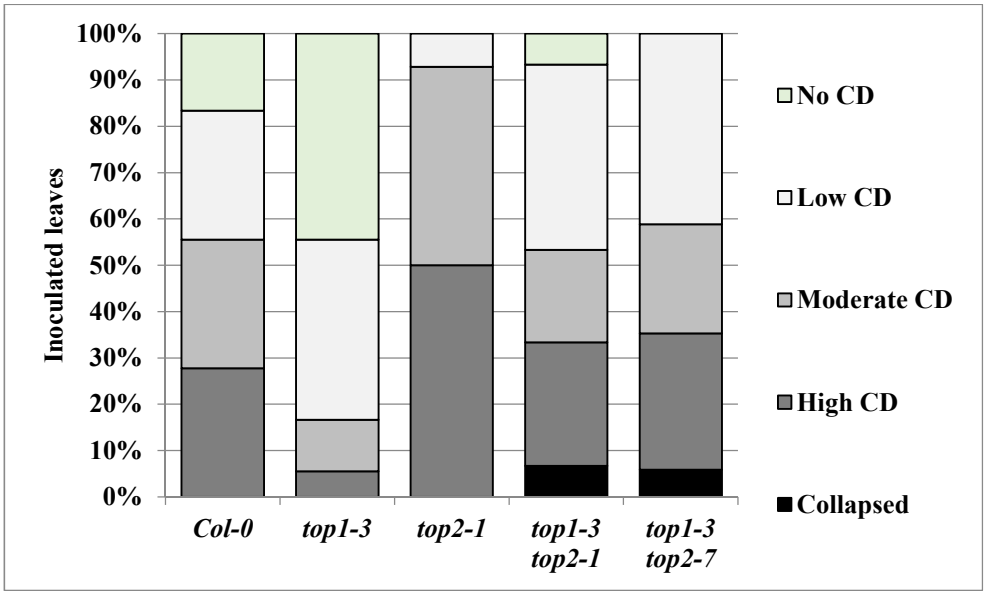
(E) Resistance towards necrotrophic pathogen *Pectobacterium carotovorum* was assessed by measuring the bacterial growth during disease progression. CFU count was analyzed 2 days post inoculation. There were no statistical differences between the lines. Data from two trials, with three replicates per treatment.

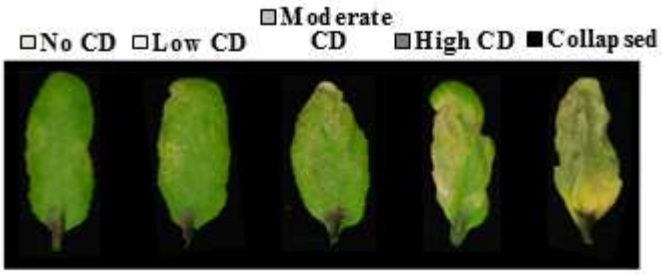
We examined whether cell death induced by necrotrophic pathogens was modulated by TOPs. Plant oxidative stress typically promotes the virulence of necrotrophic pathogens (Kim et al., 2008; Torres et al., 2006). Plant TOPs are thought to be involved in oxidative stress related processes (Polge et al., 2009). *S. sclerotiorum* induction of oxidative stress is critical to mounting an infection (Kim et al., 2008), therefore assessing cell death of *top* mutants may elucidate TOPs role in pathogen-induced oxidative stress. Cell death induced by pathogen *S. sclerotiorum* and spread of necrosis was assessed by measuring lesion diameter. The progression of *S. sclerotiorum* disease severity in the wild-type and mutant lines are similar (Figure 3-2 B). The areas of necrosis show no signs of variation between wild-type and all *TOP* mutant lines (Figure 3-2 B). After 4 DPI, lesion diameter was unable to be calculated, as the lesion size covered the entire leaf tissue in virtually all leaves (data not shown). The cell death induced by *P. carotovorum* was also examined. Cell death assessed by ion leakage assay showed no difference in ion leakage between Col-0 and the *top* lines (Figure 3-2 C).

The integrity of defense gene activation was assessed in order to determine the potential involvement of TOPs. Defense gene expression in *top* knockout lines was examined by assessing *Pathogenesis-Related 1* (*PR1*) induction during immune-activating conditions. Flagellin fragment flg22 exposure was used to determine whether *PR1* expression was modulated. The expression of *PR1* is uncompromised in *top* double mutants (Figure 3-3A). Immune elicitor flg22 induces strong *PR1* induction in Col-0 and *top* double mutant line after 24 hours (Figure 3-3A). We next examined the expression of *TOP1* and *TOP2*. *TOP1* expression is unchanged after exposure to MAMP flg22 (Figure 3-3 B). *TOP2* expression is induced by the MAMP and shows an increase in relative expression (Figure 3-3 C). The expression of *TOP1* and *TOP2* were assessed in *top* mutant background to evaluate potential co-regulation. *TOP1* expression is induced in a *top2* mutant background after exposure to MAMP elicitor (Figure 3-3 B). Furthermore, *TOP2* expression is reduced in a *top1* background (Figure 3-3 C).

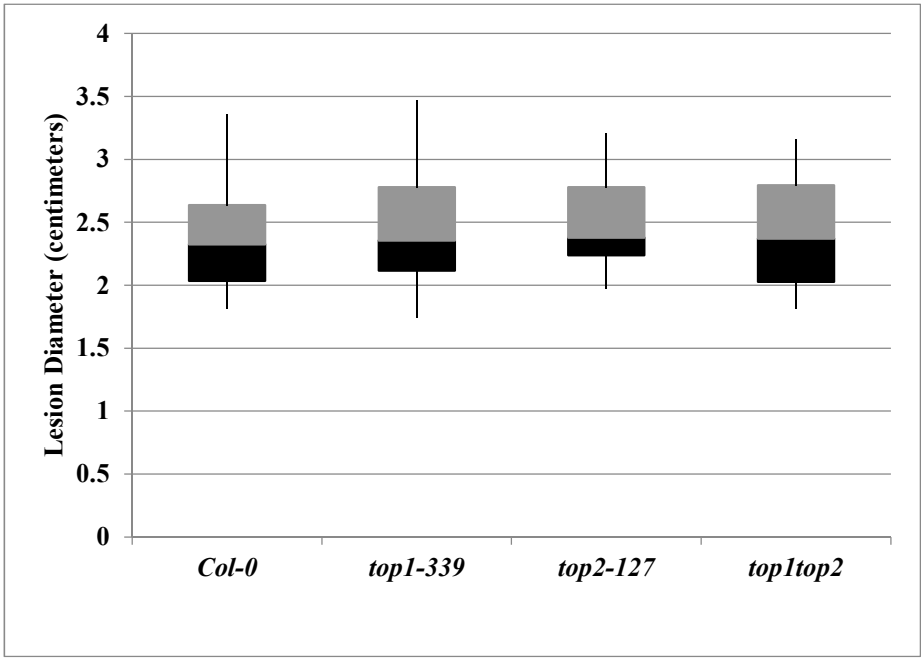
Figure 3-2

(A)

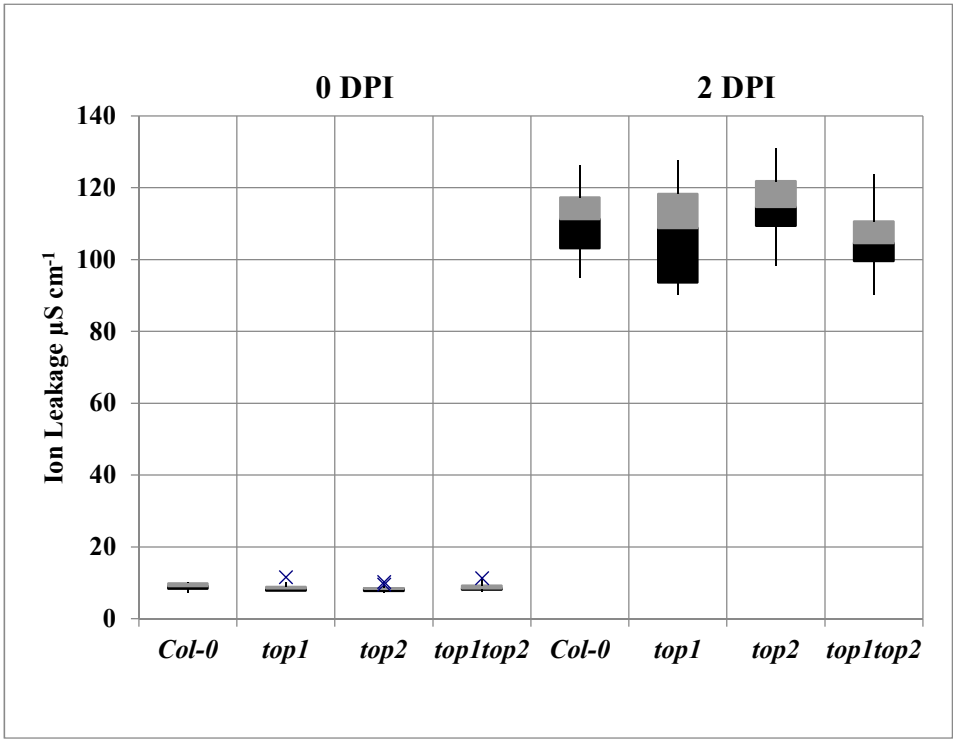

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Inoculated leaves
Col-0
top1-3
top2-1
top1-3 top2-1
top1-3 top2-7
No CD
Low CD
Moderate CD
High CD
Collapsed


No CD
Low CD
Moderate CD
High CD
Collapsed

(B)


Lesion Diameter (centimeters)
4
3.5
3
2.5
2
1.5
1
0.5
0
Col-0
top1-339
top2-127
top1top2

(C)


0 DPI
2 DPI
Ion Leakage µS cm-1
140
120
100
80
60
40
20
0
Col-0
top1
top2
top1top2
Col-0
top1
top2
top1top2

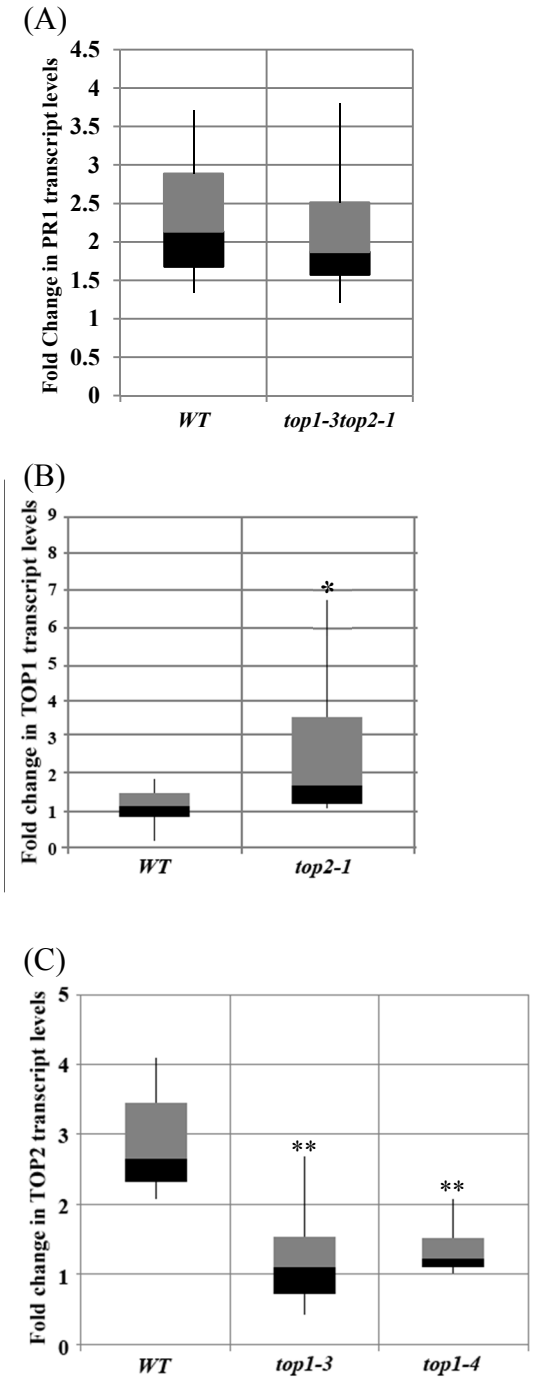
Assessing the involvement of TOP1 and TOP2 in pathogen-related cell death responses.

(A) Cell death was induced during infection with *Pst avrRpt2*. Leaves were syringe infiltrated and cell death was examined 2 days post inoculation. Leaves were detached and cell death was quantified based on the progression of symptoms. The cell death was assigned a number based on severity. *top1* exhibits less cell death relative to Col-0. *top2* lines exhibit increased amount of cell death relative to Col-0. Double mutant lines *top1-3top2-1* and *top1-3top2-7* exhibited an intermediate phenotype.

(B) Resistance towards necrotrophic pathogen *Sclerotinia sclerotiorum* was assessed by measuring the diameter of necrotic lesions during disease progression. Agar plugs of *Sclerotinia sclerotiorum* were placed on leaves. Disease symptoms were analyzed 2 days post inoculation. The cell death exhibited in the top mutant lines was similar to wild-type. There were no statistical differences between the lines (student t-test)-p value >.05. The assay was performed in two trials, with 5 replicates for each treatment.

(C) Cell death induced by necrotrophic pathogen *Pectobacterium carotovorum* was assessed by ion leakage. Leaves were inoculated with $1x10^8$ cfu/mL of *Pectobacterium carotovorum*. 8 leaf discs were floated in deionized water. Conductance was measured after initial inoculation and at 2 days post inoculation. The ion leakage readings at 2 days post inoculation indicate that the top mutant lines show no statistical differences in conductance changes from the wild-type line (student t-test)- p value >.05. The cell death in all lines show no statistical difference from mutant and wildtype lines (student t-test)-p value >.05. The assay was performed in two trials, with 4 replicates for each treatment.

Figure 3-3


(A)
Fold Change in PR1 transcript levels
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
top1-3top2-1
(B)
Fold change in TOP1 transcript levels
9
8
7
6
5
4
3
2
1
0
*
WT
top2-1
(C)
Fold change in TOP2 transcript levels
5
4
3
2
1
0
**
**
WT
top1-3
top1-4

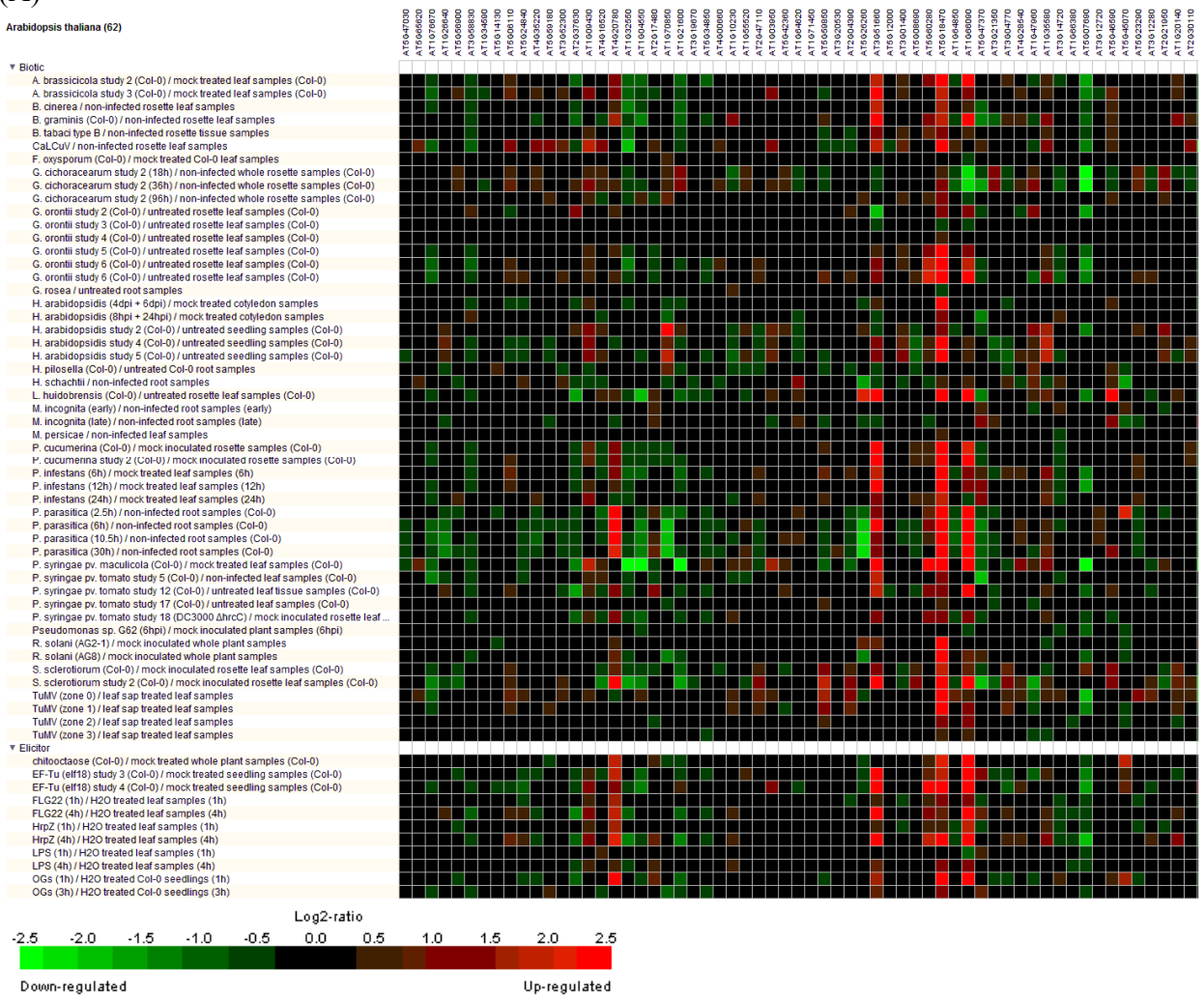
Transcriptional analysis of *PR1*, *TOP1*, and *TOP2* expression.

(A) Fold change in *PR1* expression in Col-0 and *top1-3/top2-1* double mutant lines after flg22 exposure. Data represent two combined trials. The *PR1* expression in the two lines show no statistical difference from one another (Student T-test) (p value >.05).
(B) Relative expression of *TOP1* in Col-0 and *top2-1* single mutant line after flg22 exposure. Data represent two combined trials. Asterisks represent statistical significance between Col-0 and *top2-1* mutant lines (Student's T-test) (*$p < 0.05$ and **$p < 0.01$).
(C) Relative expression of *TOP2* in Col-0 and *top1-3* and *top1-4* T-DNA insertion mutant line after flg22 exposure. Data represent two combined trial. Asterisks represent statistical significance between Col-0 and *top1* mutant lines (Student's T-test) (*$p < 0.05$ and **$p < 0.01$).

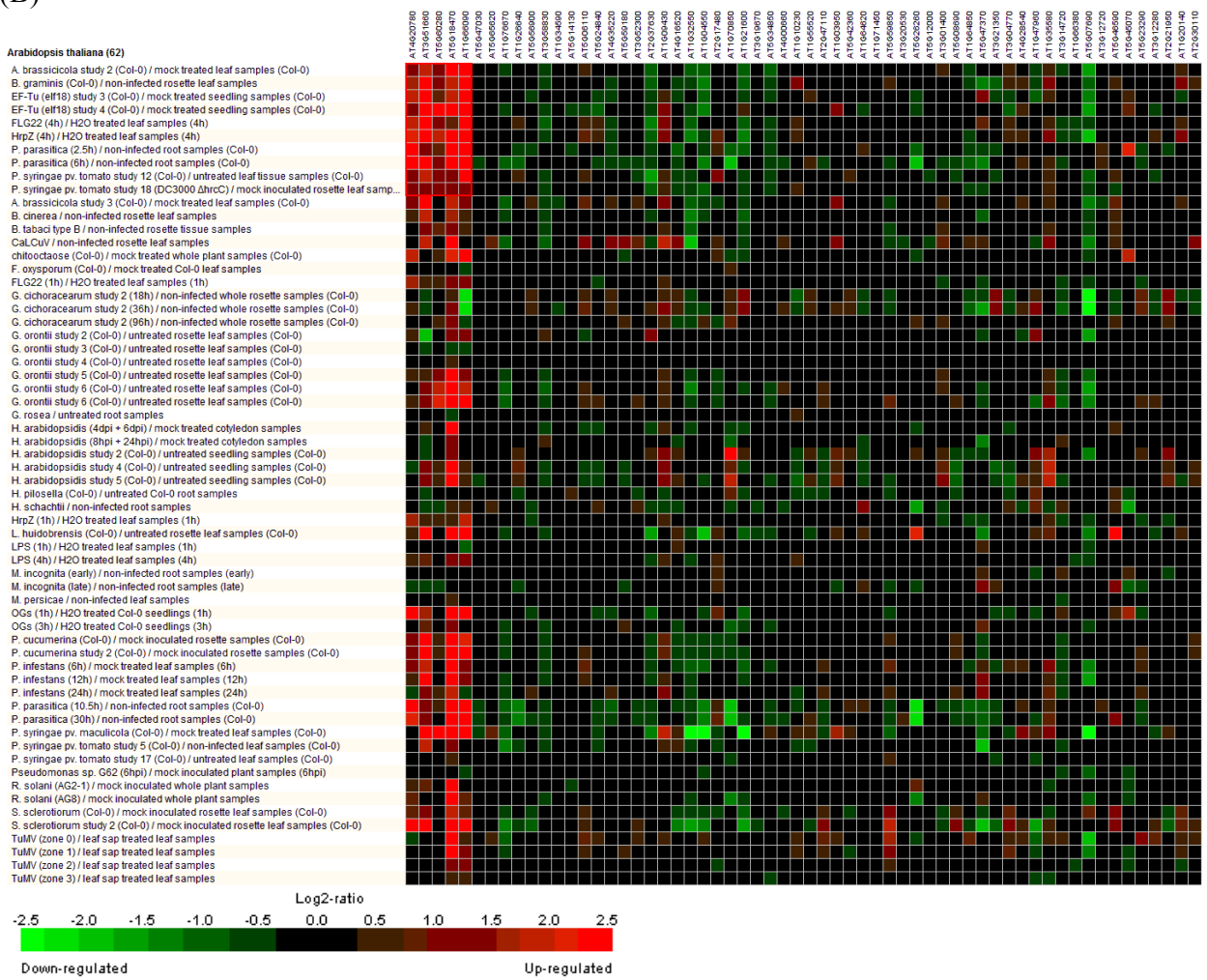
Identifying SABPs with potential roles in plant immunity and SA-regulated processes

Recently, there has been an array of SABPs identified in *Arabidopsis thaliana* (Manohar et al., 2015; Moreau et al., 2013). High-throughput screens through protein microarray have identified a plethora of potential targets of SA (Manohar et al., 2015; Moreau et al., 2013). Developing a means to select candidate SABPs for further analysis in SA-related processes is of the utmost importance. The SABPs identified through Moreau et al., 2013 protein microarray were analyzed with the meta-analysis expression database software Genevistagor (Hruz et al., 2008). Single probes were available for all but one gene (AT5G08680). Therefore, AT5G08680 was excluded from this study. The parameters for the transcriptional analysis were related to plant immunity, pathogen infections, immune-elicitors, and SA-regulated processes. The transcriptional profile of the SABPs is seen in Figure 3-4 A. BICLUSTUER co-expression and upregulation analysis showed that the following genes formed a cluster grouping; *CML42* (Calmodulin-like 42), *AtTN3* (*Arabidopsis thaliana* TIR-NBS 3), *AtMIF1* (*Arabidopsis thaliana* Macrophage migration inhibitory factor 1), *LECRK-I.8* (Lectin Receptor Kinase I.8), and *MBL-SA* (Mannose Binding Lectin-SA) (Figure 3-4 B). The highest correlated expression was during the following parameters; *Alternaria brassicicola*, *Blumeria graminis*, *Pseudomonas syringae* pv. tomato, *Phytophthora parasitica*, flg22, elongation factor EF-TU, and hrpZ (Figure 3-4 B). The five SABPs were upregulated during the immune-related processes (Figure 3-4 A, B). The sequences of the five SABPs were examined in a protein BLAST search to identify any potential sequence similarity that may indicate a conserved SA-binding site. However, no such conservation was found. NPR1's metal binding site is thought to mediate SA-binding (Wu et al., 2012). It is possible that potential metal ion sites of the SABPs are conducive to SA binding as opposed to an unambiguous SA-binding site. Utilizing the CHED server for predicting metal binding sites in proteins (Levy et al., 2009), we searched for predictive metal binding sites in the five SABPs. Calmodulin-like 42 was the only protein predicted to have a metal binding site (Table 3-2).

Figure 3-4
(A)


Arabidopsis thaliana (62)
Biotic
A. brassicicola study 2 (Col-0) / mock treated leaf samples (Col-0)
A. brassicicola study 3 (Col-0) / mock treated leaf samples (Col-0)
B. cinerea / non-infected rosette leaf samples
B. graminis (Col-0) / non-infected rosette leaf samples
B. tabaci type B / non-infected rosette tissue samples
CaLCuV / non-infected rosette leaf samples
F. oxysporum (Col-0) / mock treated Col-0 leaf samples
G. cichoracearum study 2 (18h) / non-infected whole rosette samples (Col-0)
G. cichoracearum study 2 (36h) / non-infected whole rosette samples (Col-0)
G. cichoracearum study 2 (96h) / non-infected whole rosette samples (Col-0)
G. orontii study 2 (Col-0) / untreated rosette leaf samples (Col-0)
G. orontii study 3 (Col-0) / untreated rosette leaf samples (Col-0)
G. orontii study 4 (Col-0) / untreated rosette leaf samples (Col-0)
G. orontii study 5 (Col-0) / untreated rosette leaf samples (Col-0)
G. orontii study 6 (Col-0) / untreated rosette leaf samples (Col-0)
G. orontii study 6 (Col-0) / untreated rosette leaf samples (Col-0)
G. rosea / untreated root samples
H. arabidopsidis (4dpi + 6dpi) / mock treated cotyledon samples
H. arabidopsidis (8hpi + 24hpi) / mock treated cotyledon samples
H. arabidopsidis study 2 (Col-0) / untreated seedling samples (Col-0)
H. arabidopsidis study 4 (Col-0) / untreated seedling samples (Col-0)
H. arabidopsidis study 5 (Col-0) / untreated seedling samples (Col-0)
H. pilosella (Col-0) / untreated Col-0 root samples
H. schachtii / non-infected root samples
L. huidobrensis (Col-0) / untreated rosette leaf samples (Col-0)
M. incognita (early) / non-infected root samples (early)
M. incognita (late) / non-infected root samples (late)
M. persicae / non-infected leaf samples
P. cucumerina (Col-0) / mock inoculated rosette samples (Col-0)
P. cucumerina study 2 (Col-0) / mock inoculated rosette samples (Col-0)
P. infestans (6h) / mock treated leaf samples (6h)
P. infestans (12h) / mock treated leaf samples (12h)
P. infestans (24h) / mock treated leaf samples (24h)
P. parasitica (2.5h) / non-infected root samples (Col-0)
P. parasitica (6h) / non-infected root samples (Col-0)
P. parasitica (10.5h) / non-infected root samples (Col-0)
P. parasitica (30h) / non-infected root samples (Col-0)
P. syringae pv. maculicola (Col-0) / mock treated leaf samples (Col-0)
P. syringae pv. tomato study 5 (Col-0) / non-infected leaf samples (Col-0)
P. syringae pv. tomato study 12 (Col-0) / untreated leaf tissue samples (Col-0)
P. syringae pv. tomato study 17 (Col-0) / untreated leaf samples (Col-0)
P. syringae pv. tomato study 18 (DC3000 ΔhrcC) / mock inoculated rosette leaf ...
Pseudomonas sp. G62 (6hpi) / mock inoculated plant samples (6hpi)
R. solani (AG2-1) / mock inoculated whole plant samples
R. solani (AG8) / mock inoculated whole plant samples
S. sclerotiorum (Col-0) / mock inoculated rosette leaf samples (Col-0)
S. sclerotiorum study 2 (Col-0) / mock inoculated rosette leaf samples (Col-0)
TuMV (zone 0) / leaf sap treated leaf samples
TuMV (zone 1) / leaf sap treated leaf samples
TuMV (zone 2) / leaf sap treated leaf samples
TuMV (zone 3) / leaf sap treated leaf samples
Elicitor
chitooctaose (Col-0) / mock treated whole plant samples (Col-0)
EF-Tu (elf18) study 3 (Col-0) / mock treated seedling samples (Col-0)
EF-Tu (elf18) study 4 (Col-0) / mock treated seedling samples (Col-0)
FLG22 (1h) / H2O treated leaf samples (1h)
FLG22 (4h) / H2O treated leaf samples (4h)
HrpZ (1h) / H2O treated leaf samples (1h)
HrpZ (4h) / H2O treated leaf samples (4h)
LPS (1h) / H2O treated leaf samples (1h)
LPS (4h) / H2O treated leaf samples (4h)
OGs (1h) / H2O treated Col-0 seedlings (1h)
OGs (3h) / H2O treated Col-0 seedlings (3h)
Log2-ratio
-2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
Down-regulated
Up-regulated

(B)



Expression analysis of salicylic acid (SA) binding proteins (SABPs) identified from the protein microarray.

(A) Meta-expressional analysis of the putative SABPs identified from the Moreau et al., 2013 protein microarray. The parameters utilized for the transcriptional analysis pertain to immune elicitors, SA-regulated processes, and biotic stressors.
(B) Bicluster of the SABPs to form a cluster grouping based on co-expression and upregulation during the immune-related parameters. Proteins encoded by AT1G66090, AT5G18470, AT5G60280, AT3G51660, and AT4G20780 were identified as SABPs that are co-expressed during the subset of biotic stress parameters. The five SABPs were upregulated during immune-related conditions. The threshold parameter for the analysis was .8 upregulation threshold (1.6 fold induction). Cluster groupings were derived from BiMax cluster algorithm.

The predictive localization of the five SABPs was assessed. According to TargetP, AtTN3 is predicted to contain a transit peptide and is potentially localized to the chloroplasts (Table 3-2). AtMIF1 contains a peroxisomal targeting sequence and is likely localized to the peroxisomes (Table 3-2). TargetP predicted that LECRK-I.8 and MBL-SA are secreted (TargetP) (Table 3-2). However, LECRK-I.8 is likely localized to the plasma membrane as prior studies suggest that LECRKs are plasma membrane localized (Gouget et al., 2006; Hervé et al., 1999; Navarro et al., 2003).

DISCUSSION

TOP1 and TOP2 have high amino acid sequence identity with the highest sequence identity in the peptidase domain. Additionally, we predict that the SHIFAGGY site is a peptide binding site of TOP1 and TOP2. Recent analyses of TOP1 and TOP2 activities supports that the SHIFAGGY site functions as the substrate binding site. The H and Y amino acids of SHIFAGGY site in TOP1 were found to facilitate hydrogen bonding formation to TOP substrates (Kmiec et al., 2013). Furthermore, the deletion of TOP2 SHIFAGGY site results in drastic inhibition of TOP2 activity (Chapter 2). The high sequence conservation in the peptidase domain and an identical predictive substrate binding site suggest potential shared regulation and similar substrate specificity. TOP1 and TOP2 are capable of cleaving the same synthetic substrates and are both subjected to SA control, which supports the notion that peptidases may be similarly regulated (Moreau et al., 2013).

TOP1 is predicted to possess a transit peptide (Table 3-1) and was found to be dually localized to the chloroplast and mitochondria (Kmiec et al., 2013; Moreau et al., 2013). TOP2 did not a have transit peptide (Table 3-1) and was found to be localized to the cytosol (Kmiec et al., 2013). Interestingly, a recent extensive mass spectrometry proteomic analysis has found TOP1 in the cytosolic fraction in all three independent trials (Ito et al., 2011). Proteomic studies have also identified chloroplastic TOP2 (Kleffmann et al., 2004). TOP1 and TOP2 are capable of interacting and forming heterodimers (Chapter 2). These findings suggest there may be some redundancy in TOP1 and TOP2 function and that the peptidases may potentially work cooperatively.

Table 3-2

Gene	Description	Predicted Localization (TargetP)	Peroxisomal Targeting Sequence	Predicted Metal Binding site
AT1G66090	TIR-NBS Protein	Chloroplast	None	None
AT5G18470	Mannose Binding Protein	Secreted	None	None
AT5G60280	L-TYPE LECTIN RECEPTOR KINASE	Secreted	None	None
AT3G51660	Macrophage migration inhibitory factor	Cytoplasm	Present	None
AT4G20780	calmodulin-like	Cytoplasm	None	Present

The description and predicted localization of the five SABPs that are co-expressed and upregulated during immune-related processes (AT1G66090, AT5G18470, AT5G60280, AT3G51660, and AT4G20780).

AT1G66090 is predicted to be localized to chloroplasts. TargetP analysis of AT5G18470 and AT5G60280 show that the proteins are predicted to be secreted. AT3G51660 contains a peroxisomal targeting sequence. AT4G20780 is predicted to be localized to the cytosol. Utilizing SeqCHED server metal binding site predictor, the calmodulin-like protein (AT4G20780) is predicted to contain a metal binding site (Levy et al., 2009).

TOPL1 and TOPL2 are more dissimilar to TOP1. Interestingly, evidence suggests that TOPL2 is dually localized to the chloroplast and mitochondria, like TOP1 (Carrie et al., 2015; Kleffman et al., 2004). Whether the two peptidases share similar regulation is unclear. Recent biochemical analysis suggests that like TOP1, TOPL2 can cleave transit peptides and presequences (Carrie et al., 2015). Therefore, there may be overlapping substrates in the organelles. TOPL1 does not have transit peptide and may be localized to the cytosol. It is uncertain whether TOPL1 is subject to the same form of regulation as TOP1/TOP2. TOPL1 is currently an uncharacterized M3 peptidase. The four TOP-like peptidases all contain the HEFGH active site motif. Prior sequence comparison of the TOP-like proteins with *Synechocystis* sp. PCC 6803 suggests the peptidases have ancestral cyanobacterial origins (Sokolenko et al., 2002). Cyanobacterial peptidase *slr0659* contains an HEFGH active site motif as well.

TOP1 and *TOP2* are required for a successful immune response towards specific avirulent pathogens. The integrity of the immune response is compromised in the absence of functional *TOP1* and *TOP2.* Interestingly, *top1top2* mutant lines were more resistant than *rps2* mutant lines after *Pst avrRpt2* infection. *rps2* mutant plant lines are unable to activate ETI required for resistance towards *Pst avrRpt2* infection (Yu et al., 1993). The results of the infection assays suggest that the immune response in *top1top2* mutant lines is only partially compromised. Also, PCD still occurs in *top* mutant lines, indicating ETI may still be activated. The increase in susceptibility towards avirulent pathogens may be due to the potential increase in oxidative damage in *top* mutant lines. It is noted that the susceptible phenotype occurs during the later stage of infection. *Pst* is a hemibiotrophic pathogen and induces chlorosis and necrosis at later stages of infection (Xin and He, 2013). The enhanced susceptibility seen in late stage may be related to the necrotrophic phase of *Pst*. It may be that the oxidative stress potentiated during infection results in the accumulation of potentially toxic substrates, thus, potentiating cellular damage. As TOPs are thought to be involved in oxidative stress responses, the loss of TOPs may be detrimental during the later stages of infection. Infection with necrotrophic pathogens, *P. carotovorum* and *S. sclerotiorum* showed no changes in resistance. However, the strategies that the

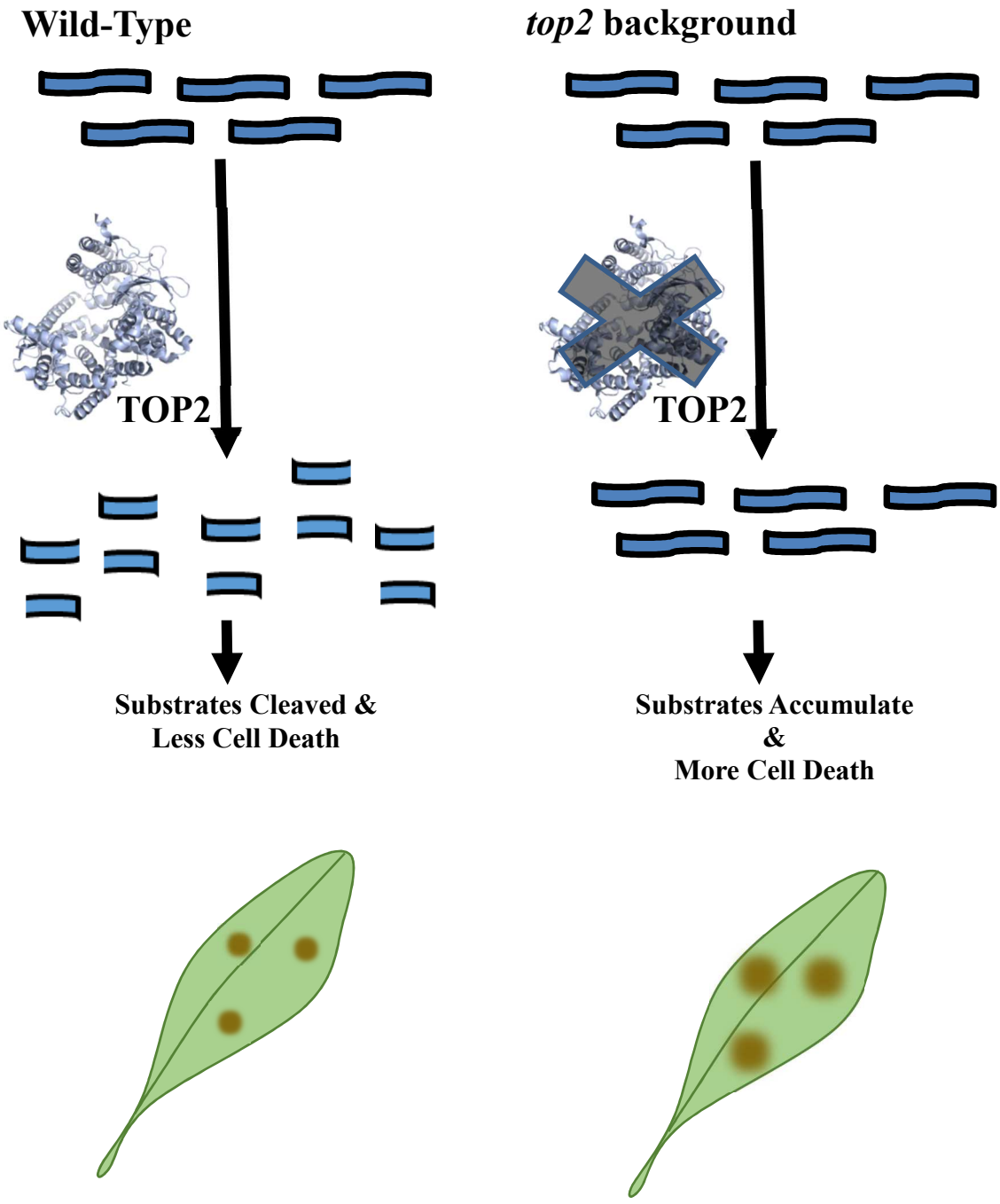
necrotrophic pathogen employs are different than hemi-biotrophic pathogens (Glazebrook, 2005). The first lines of defense between *Pst* and *P. carotovorum* are similar in that stomatal immunity is imperative, however, later stages of infection may use different immune signaling pathways (Arnaud et al., 2012; Singh et al., 2012). Thus, the lack of involvement of TOPs in *P. carotovorum* and *S. sclerotiorum* do not necessarily negate TOPs' possible role in the *Pst* necrotrophic phase. In the case of *Pst*, there is a transition between the biotrophic and necrotrophic phase. Late stages of *Pst* infection show signs of chlorosis preceded by necrosis (Xin and He, 2013) while *P. carotovorum* and *S. sclerotiorum* infections induce rapid necrosis from the start of infection.

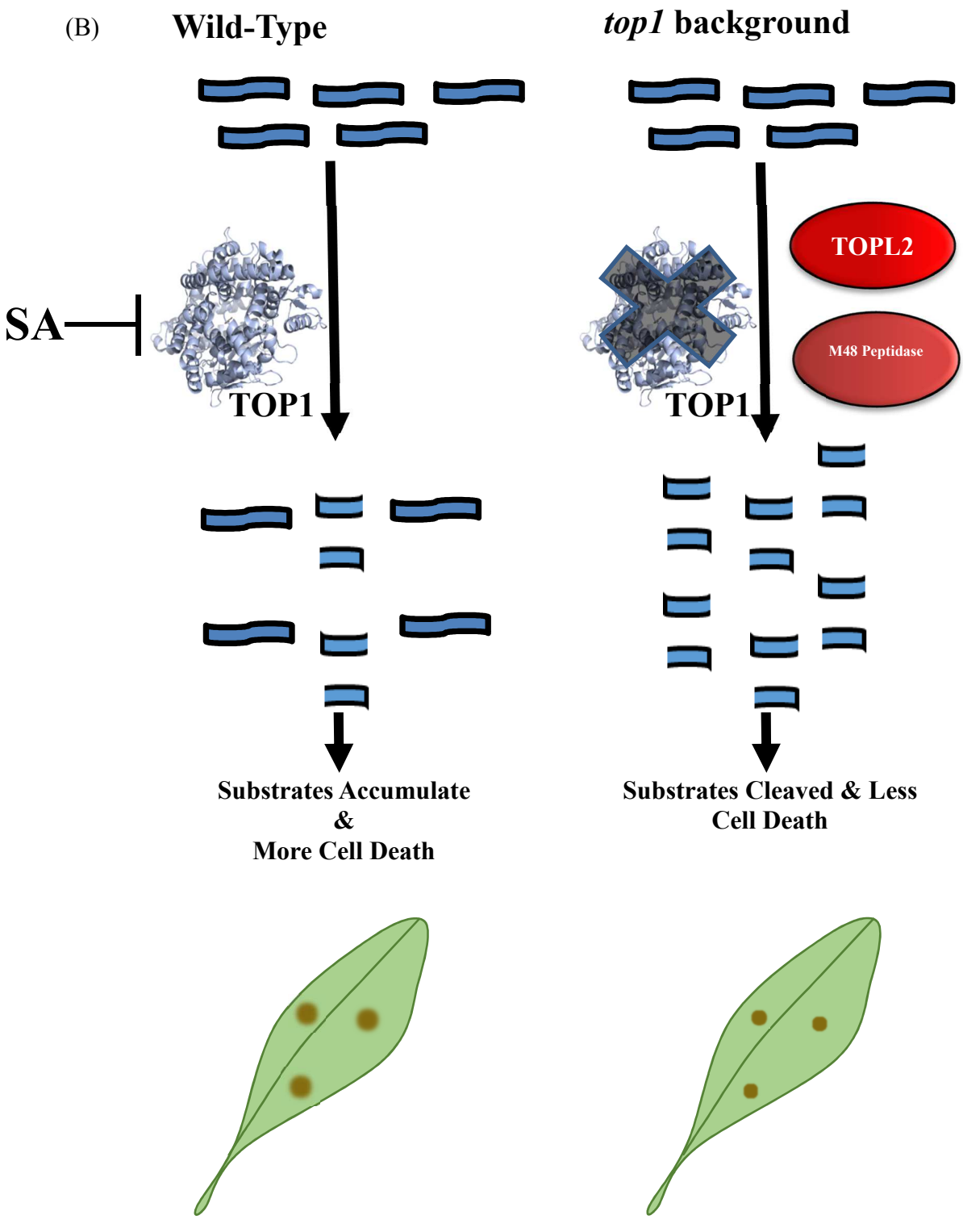
Resistance towards *Pst avrPphB* and *Pst* Δ*hrcQU* strains was not compromised by *top* mutants. *Pst avrPphB* defense activation is dissimilar to AvrRpt2 and AvrRps4 immune activation, as AvrPphB detection is thought to involve changes in the actin cytoskeletal dynamics (Tian et al., 2009). AvrPphB defense activation requires Actin-Depolymerizing Factor 4 (ADF4) (Tian et al., 2009). ADF4 is not involved in AvrRpt2-triggered resistance (Tian et al., 2009). Thus, the compromised resistance may be a result of specificity of ETI signaling. An alternative hypothesis is that specific TOP substrates modulate immune function. Mammalian TOP peptide processing is a major form of regulation in peptide hormones signaling and immunity (Dahms and Mentlein, 1992; Glucksman *et al.*, 1992; Portaro et al., 1999; Silva et al., 1999; York et al., 2003). Analogous to other systems, it may be that in the absence of TOP1 and TOP2 peptide processing, signaling from potential bioactive substrates may be impaired. Alternatively, the immune permutations may be related to ROC1, a cyclophilin, and predicted target of TOP. ROC1 is a eukaryotic activator of *Pst* effector AvrRpt2 (Coaker et al., 2006). ROC1 facilitates the active form of AvrRpt2, which in turn promotes the self-processing and cleaving of the effector (Coaker et al., 2006). In addition to ROC1's effector activation function, ROC1 is also a regulator of nucleotide-binding domain leucine-rich repeat resistant protein (NLR) activated immunity (Li et al., 2014). The cyclophilin was also found to be involved in major immune hubs such as RIN4 (Li et al., 2014). It is possible that the interaction of TOPs

with cyclophilin may influence disease resistance in an ROC1-dependant manner. Future studies are required to determine whether ROC1 is a bona fide interactor or target of TOPs.

PCD in *top* single mutants show signs of altered cell death phenotypes during infection with avirulent pathogen *Pst avrRpt2*. The *top2* lines exhibit increased cell death during infection, and the *top1* lines exhibit less death. Cell death in *top* double mutant lines shows an intermediate phenotype akin to Col-0. TOP2 is thought to be involved in oxidative stress responses. It is thought that TOP2 participates in the complete degradation of oxidized proteins (Polge et al., 2009). We postulate that TOP2 prevents the toxic accumulation of substrates. It is plausible that the accumulation of unprocessed peptides, oxidized peptides, and free peptides in *top2* may exacerbate oxidative stress and cell death (Figure 3-5 A). The reduced cell death during PCD found in *top1* lines may be due to the compensatory action of other organellar peptidases. The absence of TOP1-like oligopeptidase in other systems results in another peptidase replacing the protein's peptidase functions (Käser et al., 2003). Interestingly, a homolog of the peptidase with potential overlapping substrates is found in *A. thaliana* (Käser et al., 2003; Kwasniak et al., 2012). The homolog, an M48 peptidase encoded by At5g51740, is thought to share localization with TOP1 in the organelles (Kwasniak et al., 2012). Additionally, TOPL2 shares localization with TOP1 and may have redundant substrates. SA has an inhibitory effect on TOP1 peptidase activity (Moreau et al., 2013). It may be that in wild-type lines, SA inhibits TOP1 activity, resulting in the toxic accumulation of unprocessed peptides; this accumulation thereby contributes to PCD (Figure 3-5 B). SA may not have the same inhibitory effect on the compensatory peptidases, At5g51740 and TOPL2, in *top1* lines. Thus, PCD during infection may be limited to some extent. Alternatively, TOP1 peptide processing may regulate chloroplast homeostasis. The loss of *TOP1* may increase the accumulation of TOP1 substrates. This may cause dysfunction in particular chloroplast-related processes which in turn diminishes PCD.

Figure 3-5
(A)


Wild-Type
top2 background
TOP2
TOP2
Substrates Cleaved &
Less Cell Death
Substrates Accumulate
&
More Cell Death


(B)
Wild-Type
top1 background
SA
TOP1
TOPL2
M48 Peptidase
TOP1
Substrates Accumulate
&
More Cell Death
Substrates Cleaved & Less
Cell Death

Potential Model for TOP1 and TOP2 involvement in PCD during *Pst avrRpt2* infection

(A) In the wild-type background, TOP2 cleaves peptides to prevent the toxic accumulation of substrates and limits cellular toxicity and cell death. In *top2* background, the build-up of the TOP2 substrates potentially results in increased cell death.

(B) In the wild-type background, SA inhibition of TOP1 activity may result in an increased accumulation of TOP1 substrates. The build-up of substrates may contribute to cell death. Analogous to other eukaryotic systems, the absence of TOP-like peptidase may result in compensatory action of other overlapping peptidases. It is possible that redundant peptidases may act on TOP1 substrates in the absence of *TOP1*. SA may not impede on the activity of the redundant peptidases. Therefore, the substrates are potentially cleaved.

Cross (X) over TOP indicate mutant TOP background. 3D structural image of TOP1 and TOP2 was derived from Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al., 2000; Kanehisa et al., 2016).

Cell death induced by necrotrophic pathogens and resistance to necrotrophic pathogens does not appear to involve TOPs, as there is no differential resistance of mutant *top* lines towards *P. carotovorum* and *S. sclerotiorum*. However, since *P. carotovorum* and *S. sclerotiorum* induce rapid and robust cell death of the plant tissue, the effects of TOPs may be masked by the pronounced necrosis of the leaf tissue. Alternatively, TOPs may not play a role mediating or limiting the cell death or oxidative stress induced by *P. carotovorum* and *S. sclerotiorum*.

SA-marker gene expression was assessed to determine whether TOPs influence the integrity of immune signaling. The findings that *PR1* expression is not compromised in *top* double mutants after flg22 exposure suggesting that the upstream signaling events during MTI are unimpeded.

The expression of *TOP1* and *TOP2* was assessed in wild-type and mutant backgrounds. The expression of *TOP1* does not appear to be altered during the activation of MAMP-triggered immunity. However, the *TOP1* expression may be increased in the *top2* background during flg22 treatments. Since *TOP2* expression is induced by flg22, it may be that when TOP2 production is impeded, we may see a compensatory action resulting in increased TOP1 expression. Interestingly, the fold increase in *TOP2* expression is not seen in the *top1* mutant background during MAMP exposure. The findings suggest a form of potential co-regulation between TOPs. However, the expression is relative and not absolute. It may be that *TOP2* transcripts are already elevated in the *top1* mutant background before flg22 treatment resulting in no fold change. Future studies are required to elucidate the regulation of TOP expression.

Review of the five selected SABPs

Recently, large-scale screenings of SABPs have produced a plethora of potentially novel SA targets (Manohar et al. 2015; Moreau et al. 2013). Factors such as gene expression and localization may help find SABPs with functions related to SA and immunity. Our findings aim to develop a future pipeline in which SABPs with potential functions related to immunity are selected from large pools of proteins with SA binding properties for further analysis. Our worked examined the potential

involvement of SABPS in SA-regulated responses such as plant immunity. Utilizing Genevestigator, the expression of genes encoding the SABPs found the protein-microarray screen were analyzed. *AtTN3, LecRK I.8, AtMIF, CML42,* and an *MBL-SA* are genes with expression patterns upregulated in immune-related processes. Interestingly, the genes were found to be co-expressed and may indicate a form of co-regulation. The potential co-regulation of the SABPs may indicate a shared form SA transcriptional regulation.

AtTN3, a predicted transmembrane receptor-like protein, was found to interact with SA in the PMA. Hormone binding transmembrane receptor proteins are vital to the signaling of plant hormones cytokinin, ethylene, and brassinosteroid (Hua et al., 1998; Li et al., 1997; Suzuki et al., 2001). The predicted localization of the protein is in the site of SA synthesis and bioaccumulation. The expression analysis of the *AtTN3* gene is upregulated during a diverse array of SA-regulated processes such as pathogen infections and immune elicitors. AtTN3 is a Toll/interleukin-1 receptor (TIR) domain-containing protein. TIR domain-containing proteins are involved in the immunity of both animal and plant systems (McHale et al., 2006; Nandety et al., 2012; Takeda and Akira, 2005). In animals, the proteins are integral to innate immunity and function as immune receptors towards microbe-associated molecular patterns (MAMP) (Takeda and Akira, 2005). In plant systems, TIR domain-containing proteins have highly diversified (Meyers et al., 2002). TIR- nucleotide-binding site (TIR-NBS) is a major family of TIR-domain-containing proteins (McHale et al., 2006; Meters et al., 2002; Nandety et al., 2012). TIR-NBS proteins function in plant immunity and are involved in abiotic stress responses (Nandety et al., 2012; Zbierak et al., 2013). However, the specific involvement of AtTN3 in plant immunity is unclear.

AtMIF1 was identified as a putative SABP in the PMA. Macrophage migration inhibitory factors (MIF) are major regulators of mammalian immunity (Calandra and Roger, 2003; Hoi et al., 2006; Santos and Morand, 2006). The protein family is involved in inflammation and macrophage function (Calandra and Roger, 2003; Hoi et al., 2006; Santos and Morand, 2006). MIF functions in both mammalian innate and adaptive immune responses (Calandra and Roger, 2003; Hoi et al., 2006; Santos and

Morand, 2006). The function of MIF proteins in plants is unknown. Plants do not have adaptive immunity or a macrophage type system. In addition, the subcellular localizations of plant MIFs are unrelated to the mammalian counterparts (Srhagen *et al*., 2013). In *A. thaliana,* AtMIFs are predicted to be localized to the peroxisome or chloroplast, while the mammalian MIFs are exclusively localized to the cytosol (Panstruga et al., 2015; Srhagen et al., 2013). Interestingly, the peroxisomal MIF protein was found to interact with SA. Peroxisomes have been hypothesized to be involved in SA synthesis (Koo and Howe, 2007; Reumann et al., 2004). However, the hypothesis has yet to be confirmed. AtMIF1 is highly upregulated in plant immunity and SA-related responses. Interestingly, prior studies have shown strong induction by SA and SA analogs (Srhagen et al., 2013). The specific binding affinity of AtMIF1 to SA is unknown. There are many structurally similar phenolic molecules to SA in the peroxisomes. It may be that AtMIF1 incidentally binds to SA and may have no physiological relevance. Further studies are needed to elucidate the function and binding specificity of AtMIF1.

Calmodulin-like proteins are a class of calcium sensor proteins (Cheval et al., 2013). Ca^{2+} signaling plays a pivotal role in plant immunity and acts as an important second messenger (Cheval et al., 2013). Calmodulin-like 42 protein (CML42) was found to interact with SA and Ca^{2+} (Chiasson et al., 2005; Moreau et al., 2013). CML42 binds to three Ca^{2+} molecules (Dobney *et al.*, 2009). The binding induces conformational changes resulting in exposure of hydrophobic residues (Dobney et al., 2009). Whether the metal ion binding site facilitates SA-interactions as with NPR1 is unclear. CML42 has been implicated in trichome regulation, as the protein binds to a kinesin-interacting Ca^{+2}-binding protein that is involved in trichome development (Dobney et al., 2009). Interestingly, SA is a negative regulator of trichome production (Traw and Bergelson, 2003). It may be that SA binding to CML42 may influence trichome development. However, whether CML42 specifically binds to SA is unknown.

Plant lectins are a highly diverse class of carbohydrate-binding-like proteins (De-Schutter and Van-Damme, 2015; Lannoo and Van-Damme, 2014). Plant lectins

have immense importance in plant disease resistance and microbe recognition (De-Schutter and Van-Damme, 2015; Lannoo and Van-Damme, 2014). Lectin-domain containing proteins have been found to be involved in the perception of internal or external signals that are closely associated with disease (Lannoo and Van-Damme, 2014; Singh and Zimmerli, 2013). The protein family plays an integral role in innate immunity and responses to biotic stimuli. Plant lectins are critical in the perception of pathogens or endogenous plant-derived signaling cues which facilitate the activation of stress responses (Lannoo and Van-Damme, 2014). Many are thought to function as pattern-recognition receptors which recognize and respond to biotic stimuli (Lannoo and Van-Damme, 2014). Two lectin domain containing proteins, an MBL, and a lectin receptor-like kinase, show SA-binding capacities (Moreau et al., 2013). Interestingly, the expression of the MBL-SA and LECRK-I.8 are upregulated during diverse pathogen infections and after exposure to MAMP elicitors. LECRK-I.8 has been implicated in biotic defenses responses. Recent evidence suggests that LECRK-I.8 may be involved in SA-dependent processes such as defense activation towards *Pieris brassicae* (Reymond, 2013). The function of the proteins may be involved in cell to cell communications and signal transduction. The functional significance of SA-binding has yet to be elucidated.

Closing Remarks

TOP1 and TOP2 are two SABPs with high amino acid sequence identity. TOP1 and TOP2 mediate aspects of plant immunity and PCD. Also, recent studies have identified a plethora of proteins with SA-binding capacities. Through transcriptional analysis, we identified five SABPs whose expressions are potentially co-regulated and upregulated during plant immune-related processes, such as during biotic defense responses and immune elicitors. Future studies are required to elucidate the potential roles of the five SABPs in SA-related processes.

Supplemental Figure 3-1

TOP1 (Subject) and TOP2 (Query) Peptidase Domain Alignment- 94% sequence identity

Query Coverage 100%

```
Query       VMQHAKNRALREEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGYRNYAEVSMATK
            VMQHAKNRALREEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGY NYAEVSMA K
Sbjct       VMQHAKNRALREEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGYNNYAEVSMAMK

Query       MATVEKADELLEKLRSASWDPAVQDIEDLKSFAKNQGAAEADSLTHWDITFWSERLRESK
            MATVEKA ELLEKLRSASWD AVQD+EDLKSFAKNQGAAE+DS+THWD TFWSERLRESK
Sbjct       MATVEKAAELLEKLRSASWDAAVQDMEDLKSFAKNQGAAESDSMTHWDTTFWSERLRESK

Query       YDINEEELRPYFSLPKVMDALFGLAKTLFGIDVVPADGVAPVWNSDVRFYCVKDSSGNPT
            YDINEEELRPYFSLPKVMD LF LAKTLFGID+ PADG+APVWN+DVRFY VKDSSGNP
Sbjct       YDINEEELRPYFSLPKVMDGLFSLAKTLFGIDIEPADGLAPVWNNDVRFYRVKDSSGNPI

Query       AYFYFDPYSRPSEKRDGAWMDEVFSRSRVMAQKGSSVRLPVAQMVCNQTPPVGDKPSLMT
            AYFYFDPYSRPSEKR GAWMDEV SRSRVMAQKGSSVRLPVA MVCNQTPPVGDKPSLMT
Sbjct       AYFYFDPYSRPSEKRGGAWMDEVVSRSRVMAQKGSSVRLPVAHMVCNQTPPVGDKPSLMT

Query       FREVETVFHEFGHALQHMLTKEDEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAK
            FREVETVFHEFGHALQHMLTK+DEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAK
Sbjct       FREVETVFHEFGHALQHMLTKQDEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAK

Query       HYQTGETLPENVYKKLLAARTFRAGSLSLRQLKFATVDLELHTKYMPGGAETIYEVDQRV
            HY+TGETLPE VYKKLLAARTFRAGS SLRQLKFA+VDLELHTKY+PGG E+IY+VDQRV
Sbjct       HYETGETLPEEVYKKLLAARTFRAGSFSLRQLKFASVDLELHTKYVPGGPESIYDVDQRV

Query       SIKTQVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDDIKAVK
            S+KTQVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDDIKAVK
Sbjct       SVKTQVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDDIKAVK

Query       ETGQRFRNTILALGGGKAPLKVFVEFRGREPSPEPLLRHNG
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Sbjct       ETGQRFRNTILALGGGKAPLKVFVEFRGREPSPEPLLRHNG
```

TOP1 (Subject) and TOPL1 (Query) Peptidase Domain Alignment- 28% sequence identity

Query Coverage 96%

```
Query       RKTVAMAYGKRC--GD-TNIPVLQRLVQSRHRLACVCGYAHFADYALDRRMSKTSMRVIR
            R+ V  AY  R   GD  N  ++ ++++ R   A + GY ++A+ ++  +M+ T  +
Sbjct       REEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGYNNYAEVSMAMKMA-TVEKAAE

Query       FLEDISSSLTDLAIREFSILEDLKR--KEEGEI---PFGVEDLLYYIKRVEELQFDLDFG
             LE + S+  D A+++   +EDLK   K +G          D  ++ +R+ E ++D++
Sbjct       LLEKLRSASWDAAVQD---MEDLKSFAKNQGAAESDSMTHWDTTFWSERLRESKYDINEE

Query       DIRQYFPVNLVLSGIFKICQDLFGIKFEEVTEV-DVWYHDIRAFAVFDSGSGKLLGYFYL
            ++R YF +  V+ G+F + + LFGI  E    +  VW +D+R + V DS SG  + YFY
Sbjct       ELRPYFSLPKVMDGLFSLAKTLFGIDIEPADGLAPVWNNDVRFYRVKDS-SGNPIAYFYF

Query       DMFTR----EGKCNHSCVVALQNNALFSNGACQIPVALLIAQFAKDGSGEAVPLGFSDVV
            D ++R     G      VV+          + ++PVA ++         +   + F +V
Sbjct       DPYSRPSEKRGGAWMDEVVSRSRVMAQKGSSVRLPVAHMVCNQTPPVGDKPSLMTFREVE

Query       NLFHEFGHVVQHICNRASFARFSGLR-VDPDFREIPSQLLENWCYESFTLKLISGYRQDI
             +FHEFGH +QH+  +      +G+R ++ D  E+PSQ +ENWCY   TL  I+ + +
Sbjct       TVFHEFGHALQHMLTKQDEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAKHYE-T

Query       TKPLVDEVCKTLKRWRYSFSALKSLQEILYCLFDQIIYSD----DDADLLQLIRSLHPKV
             + L +EV K L   R   +   SL+++ +   D  +++         +  + + +  K
Sbjct       GETLPEEVYKKLLAARTFRAGSFSLRQLKFASVDLELHTKYVPGGPESIYDVDQRVSVKT

Query       MIGLPVVEGTNPASCFPRAVIGSEATCYSRLWSEVYAADIFASKFGDGHPNLYA----GL
             +  P+ E     S       G  A  YS  W+EV +AD F++    G  ++ A    G
Sbjct       QVIPPLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDDIKAVKETGQ

Query       QFRDKVLAPGGGKEPMELLTNFLGREPSTQAFI
            +FR+ +LA GGGK P+++   F GREPS +  +
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TOP1 (Subject) and TOPL2 (Query) Peptidase Domain Alignment- 27% sequence identity.

Query Coverage 99%

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            EEV +    + +S    N  ++++++  R E ++++G N+YA++ +   +A   K
Sbjct       EEVYRAYLSRASSGDLDNTAIIDQILKLRLEKAKLLGYNNYAEVSMAMKMATVEKA-AEL

Query       LQELSKTVKPKADEEFIAIRDFKREKCGNPSAELEPWDETYYTSMMKSSINDVDTAVVAS
            L++L       A ++   ++ F + +    S  +  WD T+++  ++ S  D++   +
Sbjct       LEKLRSASWDAAVQDMEDLKSFAKNQGAAESDSMTHWDTTFWSERLRESKYDINEEELRP

Query       YFPLPQCIEGLKVLVESLFGATFHTIP-LAPGESWHPNVVKLSLHHPDEGDLGYLYLDLY
            YF LP+ ++GL  L ++LFG        LAP   W+ +V    +       + Y Y D Y
Sbjct       YFSLPKVMDGLFSLAKTLFGIDIEPADGLAP--VWNNDVRFYRVKDSSGNPIAYFYFDPY

Query       SRKGKYPGCASF--AIRGGRKISE--TEYQLPVIALVCNFSRACDSSIVKLNHSEVEVLF
            SR  +  G A     +   R +++  +  +LPV  +VCN +         +   EVE +F
Sbjct       SRPSEKRGGAWMDEVVSRSRVMAQKGSSVRLPVAHMVCNQTPPVGDKPSLMTFREVETVF

Query       HEFGHALHSLLSRTDYQHFSGTR-VALDLAEMPSNLFEYYAWDYRLLKRFARHYSTGETI
            HEFGHAL  +L++ D    +G R +  D  E+PS   E + +    L   A+HY TGET+
Sbjct       HEFGHALQHMLTKQDEGLVAGIRNIEWDAVELPSQFMENWCYHRDTLMSIAKHYETGETL

Query       PEKLVNSLQGARNMFAATEMQRQVFYALIDQMLFGE----QPETARDVSHLVAELKRQHT
            PE++   L  AR   A +   RQ+ +A +D  L  +     PE+  DV   V+ +K Q
Sbjct       PEEVYKKLLAARTFRAGSFSLRQLKFASVDLELHTKYVPGGPESIYDVDQRVS-VKTQVI

Query       SWNHVEGTHWYIRFSHLL--NYGAGYYSYLYAKCFASTIWQSI----CEEDPLSLNTGTL
                +    +   FSH+    Y AGYYSY +A+  ++  + +      ++      TG
Sbjct       P--PLPEDRFLCSFSHIFAGGYAAGYYSYKWAEVLSADAFSAFEDAGLDDIKAVKETGQR

Query       LREKFFKHGGAKDPAELLTDLAGKE
             R      GG K P ++  +  G+E
Sbjct       FRNTILALGGGKAPLKVFVEFRGRE
```

TOP1 (Subject) and TOP2 (Query) N-terminus Alignment- 91% sequence identity.

Query Coverage 100%

```
Query       MASEDTLSSNPLLQNFDFPPFDSVDAHHVRPGIRALLQQLEAELEQLEKAVEPSWPKLVE
            + S++TLSSNPLLQ+FDFPPFDSVD   VRPGIRALLQ LEAELE+LEK+VEP+WPKLVE
Sbjct       VVSDETLSSNPLLQDFDFPPFDSVD---VRPGIRALLQHLEAELEELEKSVEPTWPKLVE

Query       PLEKIIDRLSVVWGMINHLKAVKDTPELRAAIEEVQPEKVKFQLRLGQSKPIYNAFKAIR
            PLEKI+DRL+VVWGMINHLKAVKDTPELRAAIE+VQPEKVKFQLRLGQSKPIYNAFKAIR
Sbjct       PLEKIVDRLTVVWGMINHLKAVKDTPELRAAIEDVQPEKVKFQLRLGQSKPIYNAFKAIR

Query       ESPDWNSLSEARQRLVEAQIKEAVLSGIALEDDKREEFNKIEQELEKLSHKFSENVLDAT
            ESPDW+SLSEARQRLVEAQIKEAVL GIAL+D+KREEFNKIEQELEKLSHKFSENVLDAT
Sbjct       ESPDWSSLSEARQRLVEAQIKEAVLIGIALDDEKREEFNKIEQELEKLSHKFSENVLDAT

Query       KKFEKLITDKKEIEGLPPSALGLFAQAAVSKGHETATADTGPWLITLDAPSYLP
            KKFEKLITDKKEIEGLPPSALGLFAQAAVSKGHE ATA+ GPW+ITLDAPSYLP
Sbjct       KKFEKLITDKKEIEGLPPSALGLFAQAAVSKGHENATAENGPWIITLDAPSYLP
```

TOP1 (Subject) and TOPL1 (Query) N-terminus Alignment

```
Query       AAKGESISPEAKCYLQCLVRDFE
            AA  ES+  +       L++DF+
Sbjct       AAAVESVVSDETLSSNPLLQDFD

Query      LIQCCVFPKMLSPHDNV
           L+Q   FP    P D+V
Sbjct      LLQDFDFP----PFDSV
Query      QNLEKTQNK
           Q LEK  +K
Sbjct      QELEKLSHK
```

TOP1 (Subject) and TOPL2 (Query) N-terminus Alignment

```
Query       PSSPEIIKAMDEISDTVCCV---VDSAELCRQTHPDREFVEEANKAAIEMNDYLHHLNTN
            P+ P++++ +++I D +  V   ++  +  + T   R  +E+     ++    L
Sbjct       PTWPKLVEPLEKIVDRLTVVWGMINHLKAVKDTPELRAAIEDVQPEKVKFQLRLGQSKPI

Query       HTLYAAVKKAEQDSNLLTKEASRTAHHLRMDFERGGIHLDPEKLDKVNNLTTNIFQLCRE
            +  + A++++  D + L++   R       +    GI LD EK ++ N +   + +L  +
Sbjct       YNAFKAIRES-PDWSSLSEARQRLVEAQIKEAVLIGIALDDEKREEFNKIEQELEKLSHK

Query       FSENIAD
```

```
            FSEN+ D
Sbjct       FSENVLD
Query       VDIFPGSR
            VD+ PG R
Sbjct       VDVRPGIR
```

Plant TOPs sequence alignments

The amino acid sequence comparison between TOP1 and TOP2, TOPL1, and TOPL2. Sequence analysis was done between the C-terminal peptidase domains and N-terminus.

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CHAPTER 4

FUTURE DIRECTIONS

Elucidating the substrates of TOP1 and TOP2

Elucidating the cellular targets of plant thimet oligopeptidases (TOPs) would yield valuable insight into the proteolytic systems TOPs may function in. Currently, the targets and pathways that TOPs are thought to function in are based on *in vitro* biochemical assays (Kmiec et al., 2013; Polge et al., 2009). In addition, whether TOPs target bioactive peptides or pathogen-derived peptides is unknown. There are multiple approaches to identifying substrates of TOPs. Development of inactive mutants may be key to determining the substrates of TOP1 and TOP2. Mutating the HEXXH active site motif renders TOPs enzymatically inactive but yet capable of substrate binding (Rioli et al., 2002). The substrates would remain bound to TOP and would be identified by mass spectrometry. Utilizing this technique, plants expressing the inactive TOP mutants could be used to determine novel targets of the peptidase. Alternatively, the peptide libraries or peptide pools can be exposed to recombinant inactive TOPs. Additionally, peptide pools may be extracted from plants during a variety of stress responses. Candidate peptides bound to TOPs would be identified for further analysis. Additionally, isobaric tags for relative and absolute quantitation (iTRAQ) mass spectrometry (MS) may also be an avenue in which substrates can be identified through comparative peptidomics. Inactive TOP mutant lines or knockout lines may result in the accumulation of TOP substrates. iTRAQ MS technique would be utilized to elucidate the differences in the peptide pools between the wild-type and mutant lines. The differences in peptide accumulation would be used to generate a list of candidate TOP targets. In addition, the plant lines may be treated with various pathogens or abiotic stressors to determine potential TOP substrates during abiotic or biotic stress.

Elucidating the mechanisms of TOP regulation

Our studies indicate that plant TOPs are subject to regulation by glutathione (Chapter 2). Glutathione induces oxidative oligomerization that results in the formation of oligomeric TOP2 (Chapter 2). However, whether S-glutathionylated

TOP2 has a function outside of inducing the multimeric form is unclear. Interestingly, in addition to the role in the formation of oligomer in mammalian systems, S-glutathionylation of TOP also results in a hyperactive form of the enzyme (Demasi et al., 2008). Whether TOP1 and TOP2 are subject to the same form of regulation is unclear. Future studies are required to elucidate the activity of the S-glutathionylated form of TOP. In addition, the cysteine residues responsible for the enzymes' interaction with glutathione are unclear. Due to the glutathione-induced thiol-exchange mechanisms, the sites that dictate glutathione binding are also sites of the intermolecular bond formation. Identification of the cysteine residues involved in glutathione binding is pivotal to understanding TOP redox-regulation.

Identifying SABPs involved in SA-signaling and SA-regulated processes

In the past decade, high-throughput screens for SA-binding proteins (SABPs) have identified over 150 proteins with the capacity to bind to SA (Manohar et al., 2015; Moreau et al., 2013). However, not all the proteins are expected to have a role in SA signaling. Utilizing transcriptional analysis programs, we have identified five promising candidate SABPs from the Moreau et al., 2013 protein microarray screen. The five genes encoding the candidate SABPs; *CML42* (Calmodulin-like 42), *AtTN3* (*Arabidopsis thaliana* TIR-NBS 3), *AtMIF1* (*Arabidopsis thaliana* Macrophage migration inhibitory factor 1), *LECRK-I.8* (Lectin Receptor Kinase I.8), and *MBL-SA* (Mannose Binding Lectin-SA are up-regulated during an array of biotic stressors and by immune elicitors (Chapter 3). In addition, the SABPs may be co-regulated which may indicate a shared form of SA-regulation (Chapter 3). However, the role of the SABPs in hormone signaling and hormone mediated responses is unclear. Further studies are needed to characterize the function of the SABPs and determine their potential involvement in SA-signaling. RNA-sequencing (RNA-seq) is a potential avenue in which the SA-responses can be assessed. Utilizing T-DNA insertion mutants or RNAi lines, the expression or production of the SABPs can be targeted. By inducing SA-signaling in the mutant lines or RNAi lines, the transcriptional reprogramming induced by SA can be assessed by RNA-seq. Comparative expression analysis may yield information on whether SA-mediated transcriptional changes are

permutated. This strategy presents a feasible approach in characterizing the involvement of the newly identified putative SABPs in SA-signaling.

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